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RESEARCH

N-myc Downstream-regulated Gene 1 (NDRG1) a Differentiation Marker of Human Breast Cancer

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Abstract N-myc downstream-regulated gene 1 (NDRG1), also called differentiation-related gene-1 (Drg1) and Cap43, is expressed in various normal tissues and suppressed in several malignancies. In this study, whether NDRG1 expression was correlated with differentiation of human breast cancer cells has been investigated. Endogenous expression level of NDRG1 was closely correlated with differentiation status of breast cancer cell lines. Furthermore, sodium butyrate (NaB), an inducer of cellular differentiation, increased the expression of β-casein, a milk-related differentiation marker, together with up-regulation of NDRG1 in breast cancer cells. In contrast, inhibition of NDRG1 by its siRNA resulted in reduced accumulation of β-casein. Immunohistochemical analysis showed co-expression of NDRG1 and β-casein or milk fat protein (MFP), another differentiation marker of breast tissue, in the mouse xenograft model of breast cancer. Furthermore, overexpression of NDRG1 expanded the differentiated area in the xenograft model of breast cancer. In human breast cancer, using samples from 45 patients, we also showed close relationship between NDRG1 and β-casein or MFP expression. Altogether, in vitro and in vivo data demonstrated a possible role of NDRG1 in differentiation of breast cancer. We concluded that NDRG1 could be used as a biomarker for differentiation of breast cancer for both diagnostic and therapeutic purposes.

Keywords Breast cancer · Differentiation · Cancer biomarkers · NDRG1 · Cap43 · Anticancer differentiation therapy

Abbreviations

NDRG1	N-myc downstream-regulated gene 1
MFP	Milk fat protein
ATRA	All-trans-retinoic acid
HDAC	Histone deacetylase
Rit42	Reduced in tumor 42 kDa

Introduction

N-myc downstream-regulated gene 1 (NDRG1) is a nickel- and calcium-inducible gene [1–3]. The product of this gene

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is a 43-kDa protein with three unique 10 amino-acid tandem-repeat sequences at its carboxyl terminus. Expression of *ndrg1* gene is highly susceptible to various stimuli including oxidative stress, metal ions, hypoxia, as well as oncogenes such as *n-myc* and *c-myc* and tumor suppressor genes such as p53 and VHL [3–8]. NDRG1 is expressed in various organs including prostate, ovary, colon and kidney, and its expression is dynamically changed during the postnatal development in kidney, brain, liver and nerves [3, 9–11]. Involvement of NDRG1 in organ maturation and differentiation has been studied in neuronal system. Okuda et al. have generated NDRG1-deficient mice [12]. Targeted destruction of NDRG1 induces Schwann cells dysfunction, suggesting that NDRG1 is essential for maintenance of the myelin sheaths in peripheral nerves [12]. NDRG1 also plays an important role in the terminal differential of Schwann cells during nerve regeneration [13]. Expression of NDRG1 is reduced in tumor cells. Therefore, it is also called "reduced in tumor 42 kDa" or Rit42 [4]. In contrast, overexpression of the NDRG1 gene in the animal model inhibited the growth of colon cancers and prevented the metastasis of prostate and colon cancer cells [14, 15]. In colon and prostate cancers, expression of the NDRG1 gene is up-regulated in normal tissue and highly differentiated cancer cells, but down-regulated in poorly differentiated cancer cells [14, 15]. Low expression of NDRG1 is correlated with poor clinical outcome in breast cancer [16]. We have also shown that expression of NDRG1 is down-regulated upon estradiol stimulation, and its expression is correlated with favorable prognosis in breast cancer patients [17]. On the other hand, induction of differentiation is considered a promising alternative or complementary to standard anti-cancer chemotherapy. This type of treatment has the advantage of being potentially less toxic than other agents used in standard protocols of chemotherapy [18]. All-trans-retinoic acid (ATRA), phorbol ester, histone deacetylase (HDAC)-inhibitors are commonly used as differentiation inducers [19–22]. Interestingly, most of these differentiating agents could often up-regulate expression of NDRG1 in various cell types [23]. Among above-mentioned agents, HDAC inhibitors have recently emerged as promising anticancer therapeutics [22]. The anti-tumor activity of HDAC inhibitors has been linked to altered gene expression through modification of chromatin structures [24]. Sodium butyrate is one of the HDAC inhibitors that induces differentiation in various cells, including leukemia [25–28]. We have previously demonstrated that expression of NDRG1 is highly susceptible to estrogen receptor activity in human breast cancer cells in culture and cancer patients [17]. In this study, we examined the relationship between NDRG1 expression and differentiation in human breast cancer cells and tissues. Furthermore, we will discuss if NDRG1 could be a novel target as well as a molecular marker for differentiation inducing anticancer therapeutics in breast cancer.

Materials and Methods

Cells and Cell Culture Human breast cancer cells SK-BR-3, MDA-MB-231, T47D, MCF-7 were obtained from the American Type Culture Collection (ATCC) and were grown in McCoy, DMEM, RPMI and α-MEM media, containing 10% fetal bovine serum (FBS), 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate.

Expression Vector Construction and Transfection NDRG1 expression vector construction and transfection was performed as previously explained [29]. Briefly, NDRG1 cDNA was amplified by reverse transcription-PCR using the 5′ and 3′ primers 5′-CATGTCTCGGGAGATGCAGGATG-3′ and 5′-AGGCCGCCTAGCAGGAGACC-3′, respectively. Amplified NDRG1 cDNA was ligated into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) and transferred to the pIRESneo2 expression plasmid (pIRESneo2-NDRG1). MDA-MB-231 cells were transfected with pIRESneo2-NDRG1 or pIRESneo2 using LipofectAMINE 2000 (Invitrogen) following the manufacturer's protocol. Stable transfected clones were isolated by incubation with G418 selection.

Animal Experiments Male athymic nu/nu mice, 5 weeks of age, weighing 21 to 27 g, and specific pathogen–free, (purchased from Charles River Laboratories, Yokohama, Japan) were inoculated with either parental or mock or NDRG1 stable MDA-MB-231 cells suspended in 100 μL sterile PBS at a concentration of 10^8 cells/mL. Animals were observed for signs of tumor growth and activities. All animal experimental procedures were approved by the Committee on the Ethics of Animal Experiments in JoyUp Biomedical, Co. Ltd. Fukuoka, Japan.

Immunocytochemistry Cells were cultured in their specific media containing 10% fetal bovine serum. Then they were trypsinized and plated on glass coverslips in 6-well plates and allowed to attach overnight. Then, cells were rinsed with phosphate-buffered saline (PBS) at room temperature and then fixed with 4% paraformaldehyde/PBS for 30 min at RT. Cells were rinsed twice with PBS and then permeabilized with 0.5 ml of solution containing 5% bovine serum albumin (BSA), 0.2% Triton X-100 in PBS for 90 min at RT. After 1 h of blocking with 2% goat serum, the cells were incubated overnight with rabbit polyclonal anti-NDRG1 (1:1000, developed in our institute) mouse monoclonal anti β-casein (F20.14) antibody (Abcam, 1:100), mouse anti-milk fat (globulin) protein

(MFP, Chemicon International, 1:100) at 4°C in 1% BSA in PBS. Cells were then rinsed three times with PBS and, based on host of the primary antibody, were incubated with goat anti-rabbit IgG; 1 μg/ml Alexa Flour 546 (or 388) or anti-mouse IgG; 1 μg/ml Alexa Flour 388 (or 546) (Molecular Probe, Oregon, USA) in 1% BSA in PBS for 60 min at RT. 4′ 6-Diamidino-2-phenylindole (DAPI) (1:1000, Dojindo, Japan) was used for nuclear staining. Cover-slips were mounted on slide glasses using gel mount and viewed using a Olympus BX51 florescence microscope and photographed with Olympus DP-70 digital camera (Olympus, Japan).

Lipid Assay by Oil Red O and Nile Red Staining Oil Red O and Nile red staining was used to visualize neutral lipids in cells. Oil red O staining was performed as follow: after removing the medium, cells were fixed in a solution of 10% formamide containing 1% $CaCl_2$. After washing in water, cells were stained in Oil Red O Solution (0.3% Oil Red O in 60% isopropanol) for 15 min at room temperature. Slides were then rinsed with deionized water and counterstained with hematoxylin blue. Red staining of neutral lipids was examined by transmitted light microscope (Olympus, Japan). Nile red staining procedures were similar to Oil red O staining except final step that Nile red stained cells were viewed using an Olympus BX51 florescence microscope (Olympus, Japan). Stained cells were photographed using Olympus DP-70 digital camera (Olympus, Japan).

NDRG1 siRNA Down-regulation SK-BR-3 cells were plated in 6-well culture plates (3×10^5 cells per well) 24 h prior to transfection. Cells were transfected with either 50 nM control or NDRG1 siRNA (purchased from Qiagen, Maryland, USA) using RNAiMAX (Invitrogen). Following transient transfection, levels of NDRG1 were evaluated by immunoblotting.

Western Blotting Cells were plated and grown in 6-well plates at ~50% confluency. The cells were harvested with a rubber policeman, and the cell slurry was sonicated briefly before centrifugation at 15,000*g* for 15 min at 4°C. The supernatant was collected, and aliquots of proteins were loaded into each well and separated on SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Separation, blotting, and visualization of the proteins were performed according to Western blotting protocol. Antibodies used were as follows: rabbit polyclonal antibody directed against NDRG1 (produced in our institute), mouse anti-β-actin (Sigma) and rabbit anti-GAPDH antibodies (Santa Cruz Biotechnology).

Immunohistochemical Analysis Tissue sections from 45 breast cancer patients who underwent radical surgery between 1995 and 1999 at Department of Surgery, Kurume University Hospital, were used for immunohistochemical study on human breast cancer. After routine de-paraffination and rehydration through gradient ethanol immersions, the slides were steam-heated in citrate buffer for 20 min for antigen retrieval. Endogenous peroxidase activity was quenched using 3% (v/v) H2O2 followed by three 5-min washes in PBS containing 0.2% (v/v) Triton X-100, and the sections were blocked with 10% (v/v) normal goat serum in PBS. Specimens were incubated for 1 h with either NDRG1 or milk fat (globulin) protein (MFP) (Chemicon International Inc.) antibodies diluted in PBS containing 0.3% (v/v) Triton X-100 and 0.1% (w/v) bovine serum albumin. Specimens were counterstained with diluted hematoxylin for 30 s and washed with tap water. Then, the sections were mounted with glycerin (Dako, USA) and coverslips. Images were obtained using an Olympus BX51 florescence microscope and photographed with Olympus DP-70 digital camera (Olympus, Japan). Similar protocols were applied for studying on paraffin-embedded samples from the xenograft model. For florescent immunohistochemistry, frozen sections from 15 breast cancer tissues from above-mentioned archive were fixed in freshly prepared 4% PFA and double-immunostained for NDRG1 together with MFP or β-casein. Secondary antibodies and florescence microscopy used for viewing the slides were same as methods described for immunocytochemistry. Similar protocols were also used for frozen samples from xenograft models.

Image Analysis Image-Pro Plus, Version 6 for Windows (Media Cybernetics, Inc., MD, USA) was used to analysis signal intensity or percentage of overlay of various signals. Co-expression of NDRG1 with β-casein or MFP in vivo or in vitro condition, as well intensity of Oil Red O staining was evaluated using this software.

Results

NDRG1 Expression in Human Breast Cancer Cell Lines and Their Differentiation Status Four breast cancer cell lines were analyzed for NDRG1 expression. The relationship between NDRG1 levels and differentiation status of the representative cell lines was also evaluated (Fig. 1). Among four cell lines, SK-BR-3 and MDA-MB-231 showed relatively higher, and MCF-7 and T47D showed lower expression of NDRG1 (Fig. 1a). To evaluate the relationship between the differentiation status and NDRG1 expression, SK-BR-3 and T47D cell lines, as representatives, were analyzed for neutral fat contents, using Oil red O and Nile red staining (Fig. 1b) and immunostaining for

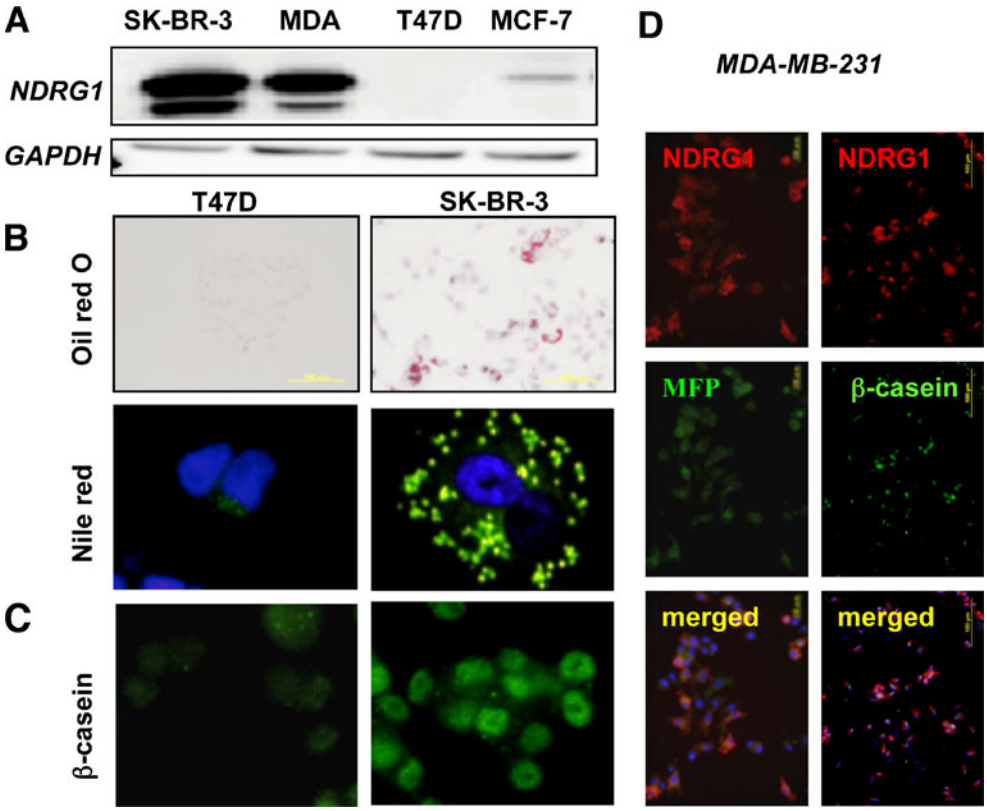

Fig. 1 NDRG1 expression in breast cancer cell lines and its relationship with differentiation status. **a** Four breast cancer cell lines were analyzed for NDRG1 expression. GAPDH was used as a control for equal loading. **b–c** The differentiation status of SK-BR-3 with higher NDRG1 expression was compared to T47D with lower NDRG1 expression. Cellular neutral fat contents (**b**), demonstrated with Oil red O (*upper panel*) and Nile red staining (*lower panel)* and (**c**) β-casein contents were used for evaluation of differentiation status. For Nile red staining, the cellular nuclei were stained with DAPI. **d** The association between NDRG1 expression and differentiation status in individual cells was demonstrated by double-staining of MDA-MB-231 cells for NDRG1 and milk fat globulin (MFP) and β-casein, two milk-production related differentiation markers of breast tissue

β-casein (Fig. 1c), as differentiation-related factors. We observed higher intensity of Oil red O and Nile red staining and β-casein expression in SK-BR-3 than T47D cells, indicating that SK-BR-3 is relatively more differentiated (Fig. 1b, c). To further demonstrate the association between NDRG1 and differentiation status, MDA-MB-231 cells were double-stained for NDRG1 and milk fat protein (MFP) and β-casein (Fig. 1d). There was a close co-expression of NDRG1 and MFP and β-casein in some cell populations of MDA-MB-231 cells.

In Vitro Differentiation of Breast Cancer Cells Up-regulates NDRG1 Expression Data presented in Fig. 1 indicated a close association between NDRG1 expression and differentiation status of breast cancer cells. To further evaluate this relation, we induced cellular differentiation using HDAC inhibitor. Four breast cancer cell lines used in this study were incubated with 0, 2 or 4 mM of sodium butyrate (NaB) for 5 days. Then, the cells were washed briefly with PBS and their neutral fat contents were stained with Nile red or Oil red O. Representative images of cells treated with 2 mM are shown (Fig. 2a). Non-stimulated SK-BR-3, and MDA-MB-231 cells showed significantly higher number of both Oil red O and Nile red stained cells, compared with MCF-7 and T47D. Furthermore, the number of Nile red and Oil red O stained cells was significantly increased after incubation with NaB in all cell lines ($p<0.05$, Fig. 2b). This indicates that all cell lines are susceptible to differentiation induced by 2 mM NaB. To evaluate the changes in NDRG1 expression following induced differentiation we measured protein level in control and NaB-treated cells. Western blot analysis of cells incubated with NaB showed increasing expression of NDRG1 protein in a dose dependent manner (Fig. 3a). Although expression levels of NDRG1 protein were much less in estrogen-positive-MCF-7 and T47D, there were apparent increases in NDRG1 expression in response to NaB. Accordingly, immunocytochemistry (Fig. 3b) also showed an increased number of NDRG1/β-casein-positive cells in NaB treated compared to control ($p<0.05$, Fig. 3c).

Down-regulation of NDRG1 Reduced Cellular Differentiation In Vitro Association between NDRG1 and natural as well as induced differentiation raises the question whether down-regulation of endogenous NDRG1 expression could affect cellular differentiation. To address this, expression of NDRG1 in SK-BR-3 was inhibited by NDRG1 siRNA. NDRG1 expression in SK-BR-3 cells was effectively down-regulated after treatment with 50 nM of NDRG1 siRNA for 48 h (Fig. 4a). In contrast, double-immunostaining of control and NDRG1 siRNA-treated cells showed a significant decrease in expression of β-casein upon NDRG1 down-regulation ($p<0.01$, Fig. 4b–c).

Relationship between NDRG1 Expression and Differentiation In Vivo In vitro data suggested an important role for NDRG1 in cellular differentiation. To determine the effect

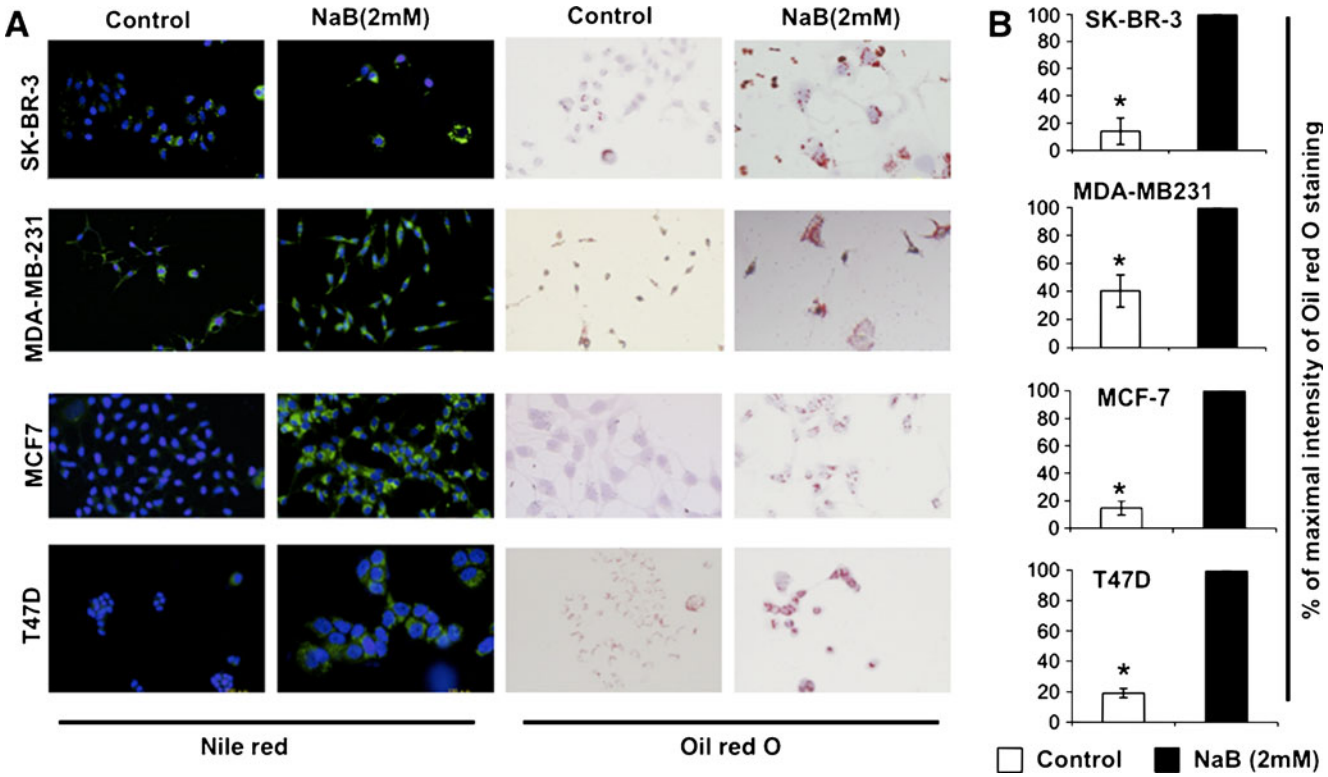

Fig. 2 Cellular Differentiation by HDAC inhibitor sodium butyrate: Four breast cancer cell lines were treated with 0, 2 and 4 mM of sodium butyrate (NaB) for 5 days. **a** The results of cells treated with 2 mM are shown. The induction of differentiation was demonstrated by staining neutral fat contents of the cells with Nile red (Fluorescent microscope) and Oil red O (phase contrast microscope). For Nile red staining, the cellular nuclei were stained with DAPI. **b** Intensity of Oil red O staining was evaluated using Image-Pro Plus image analysis software and the results are presented as% of maximal intensity of Oil red O staining (*significantly different compared to NaB-treated cells, $p<0.05$)

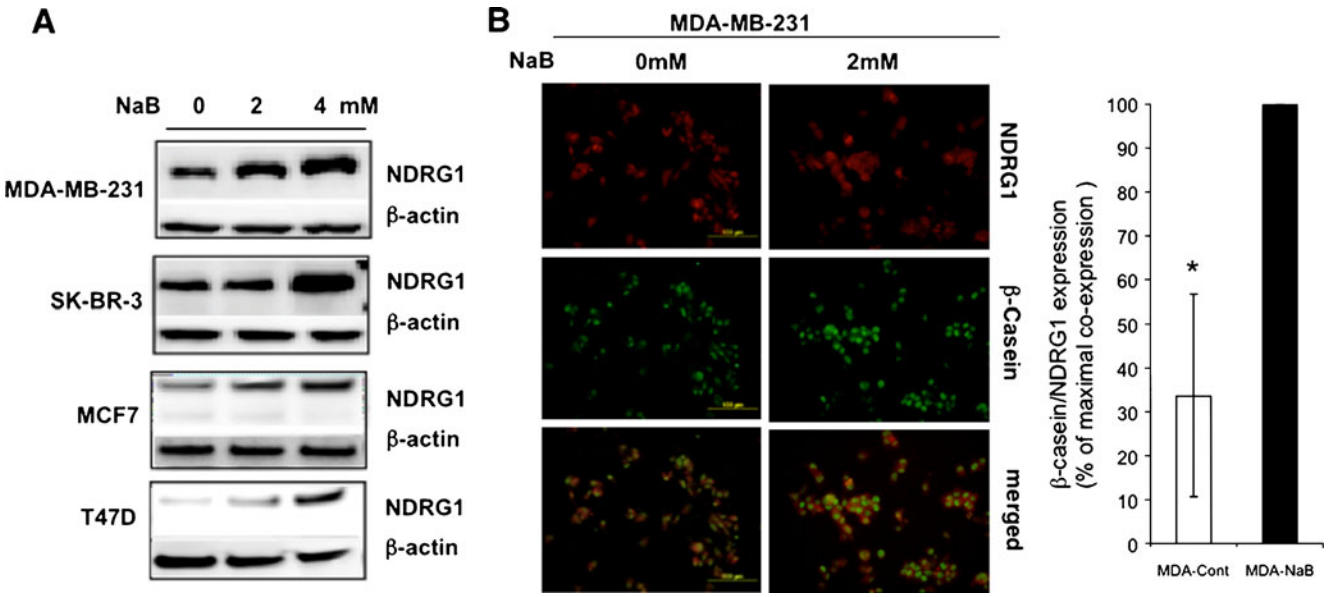

Fig. 3 NDRG1 expression was increased along with NaB-induced differentiation: **a** Four breast cancer cell lines were treated with 0, 2 mM and 4 mM of NaB for 5 days. Thirty microgram of total protein lysate of treated cells were used for immunoblotting for NDRG1. β-actin expression was used as load control. Dose-dependent increase of NDRG1 was evident in all cell lines. **b** To demonstrate the relationship between the increased NDRG1 expression in NaB-treated cells with the change in their differentiation status, MDA-MB-231 cells were immunostained for both NDRG1 and β-casein. **c** The increased number of cells simultaneously expressing β-casein and NDRG1 was evident in NaB (2 mM) treated cells ($p<0.05$)

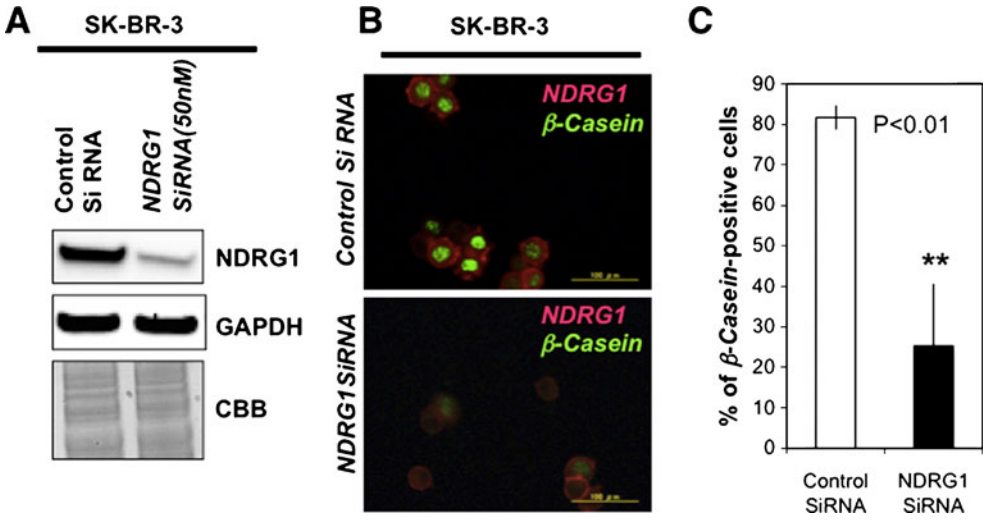

Fig. 4 Down-regulation of NDRG1 reduced the differentiation status of the cells. **a** High NDRG1 expressing SK-BR-3 cells were treated with control siRNA and NDRG1 siRNA(50nM) as it is described in Materials and Methods. Down-regulation of NDRG1 was demonstrated by immunoblotting. GAPDH immunostaining and Commassie staining of the gel were used as control for equal loading. **b** Double staining of control and NDRG1 siRNA-treated cells for NDRG1 and β-casein is shown. **c** Image analysis showed the simultaneous reduction of β-casein and NDRG1 following NDRG1 siRNA treatment (**significantly lower compared to control siRNA, $p<0.01$)

of NDRG1 on differentiation in vivo, we developed xenografts of cells with endogenous and induced expression of NDRG1 and evaluated them for differentiation criteria. To evaluate the effect of increased expression of NDRG1 on differentiation in vivo, we developed xenograft of cells with induced NDRG1 over-expression. MDA-MB-231 cells were transfected with either NDRG1 over-expressing (MDA-NDRG1) or empty vector (MDA-Mock)

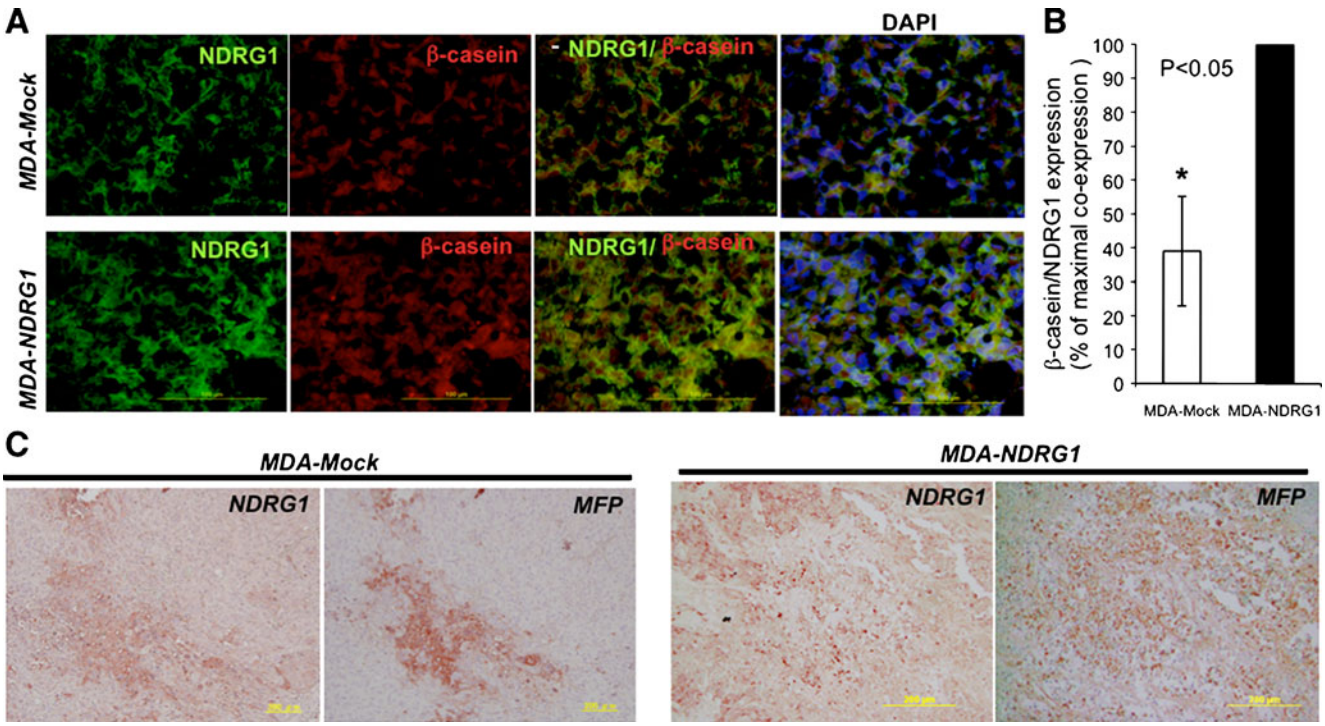

Fig. 5 Relationship between NDRG1 and differentiation status of tumor cells in vivo. MDA-MB-231 cells transfected with mock (MDA-Mock) or vector containing NDRG1 (MDA-NDRG1) were inoculated into the nude mice. Tumor masses were excised and 5 μm serial sections were prepared from frozen (**a**–**b**) and paraffin-embedded samples (**c**). **a** Frozen section of MDA-Mock and MDA-NDRG1 were stained with NDRG1 and β-casein. **b** Co-expression of NDRG1 and β- casein was analyzed using Image-Pro Plus image analysis software. The values are presented as% of maximal co-expression (*Significantly lower than MDA-NDRG1, $p<0.05$). **c** Paraffin-embedded samples were imuunostained for NDRG1 and MFP. The results for mock were compared with NDRG1 over-expressing cells

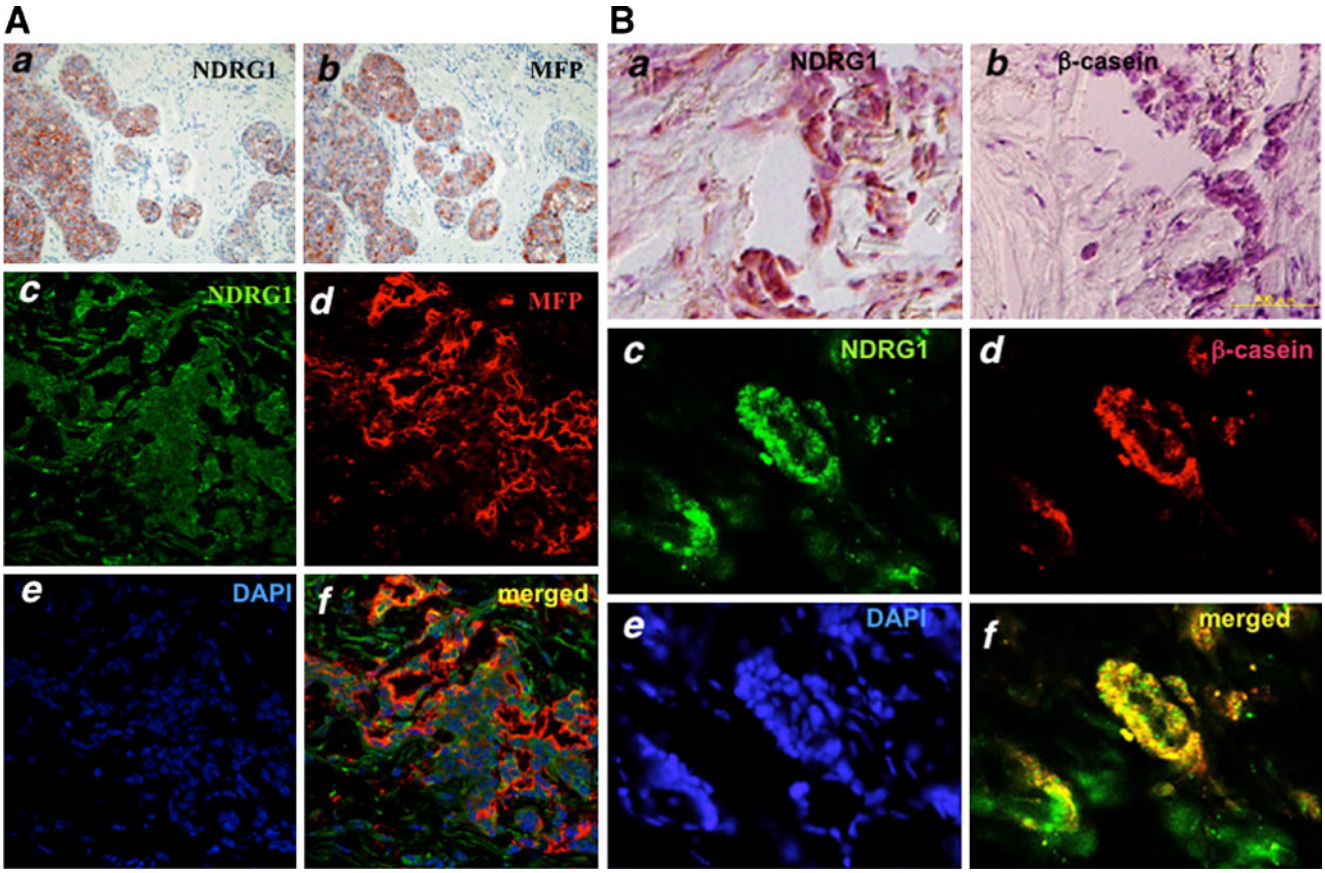

Fig. 6 Relationship between the NDRG1 expression and milk-production proteins in human breast cancer. For evaluation of the association between NDRG1 and the breast cancer differentiation, samples derived from human breast cancer patients were immunostained for NDRG1 and milk production-related breast cell differentiation markers. **a** Immunostaining of paraffin-embedded serial sections for NDRG1 and MFP (**a** and **b**) and double staining of frozen sections for both factors (**c–f**). **b** Frozen samples of breast cancer patients were also immunostained for NDRG1 and β-casein; separately (**a–b**) and together (**c–f**)

and selected confirmed clones were inoculated into nude mice and the differentiation status of xenografts excised from these mice was evaluated. Frozen sections from these xenografts were stained with NDRG1 and β-casein (Fig. 5a). Similar to the above-mentioned close association between NDRG1 and β-casein expression in xenograft from intact parental MDA-MB-231 cells, there was a close association between NDRG1 and β-casein expression in both MDA-Mock and MDA-NDRG1 cells. Furthermore, image analysis showed an increased co-expression of NDRG1 and β-casein upon NDRG1 up-regulation ($p<0.05$, Fig. 5b). Serial sections from MDA-Mock and MDA-NDRG1 xenografts were stained for NDRG1 and MFP. Representative sections are shown. In xenografts from mock transfected cells, there was a close relationship between NDRG1 and MFP-expressing areas (Fig. 5c). However, in xenografts from MDA-NDRG1 cells, NDRG1-expressing areas were significantly increased. The extensive expression of NDRG1 was also associated with increased MFP expression (Fig. 5c).

Relationship between NDRG1 Expression and Milk-related Proteins in Human Breast Cancer To evaluate the relationship between expression of NDRG1 and differentiation in human breast cancer, serial paraffin sections from the patients were immunostained with NDRG1 and MFP. Expression of both factors from randomly selected areas from each patient was evaluated and their correlation was determined. There was a significant association between areas simultaneously expressing two proteins ($p<0.01$) (Fig. 6a: *a–b*). Furthermore, double-staining of frozen samples derived from 16 patients also showed a considerable overlap between areas expressing these two factors (63.1+/−16.4% of merged areas) (Fig. 6a: *c–f*). Additionally, these frozen sections were also double stained for NDRG1 and β-casein (Fig. 6b). The areas expressing β-casein were also closely stained for NDRG1 (Fig. 6b: *a–b*) and a similar close overlay (81.7+/−10.0% of merged areas) was observed in double immunostaining (Fig. 6b: *c–f*). From IHC analysis, NDRG1 expression showed a close association with MFP and β-casein, milk production-related differentiation marker proteins of mammary glands.

Discussion

Differentiation of breast tissue and its relationship with breast cancer has been studied intensively [30–33]. This includes morphological changes during normal differentiation of breast tissue. In general, milk secretion is considered a key function indicating differentiated state of the mammary alveolar cells. Therefore, there are several milk production-related proteins considered as luminal differentiation markers, including milk proteins such as caseins, milk fat globulin (MFP), α-lactalbumin and whey acidic protein (WAP) [34]. Since these are mostly indicators of terminal differentiation, there is still a need for biomarkers indicating earlier stages of differentiation.

The relation between NDRG1 and differentiation in cancer was first reported in colon cancer [2]. The results showed that in the normal colon, NDRG1 protein (called Drg1 in that study) was expressed in the cytoplasm and basolateral membranes of surface epithelial cells that border the gut lumen, i.e., differentiated areas [2]. Although differentiation grade is an important prognostic factor for colorectal tumors, its usefulness is limited since its predictive value for tumor behavior is not very significant [35]. In contrast, differentiation stage is considered as important criteria in determination of prognosis in breast cancer [36, 37]. Current histological grading of breast cancer, which is based on the evaluation of three morphologic features: tubule formation, nuclear pleomorphism and mitotic count, is the main approach to describe proliferation and differentiation and determine prognosis in this malignancy [38]. However, there is an increased demand for reliable supportive molecular biomarkers to further evaluate these features 38. The results presented in this study showed a close relationship between NDRG1 expression and differentiation status of breast cancer cells and tissue. These suggested NDRG1 as a milk-production independent marker of differentiation which could be considered along with the histological grading. In addition to supportive pathological marker of differentiation, we also evaluated the therapeutic value of NDRG1. Drug-induced differentiation using an HDAC inhibitor such as NaB was associated with up-regulation of NDRG1 in all cell lines used in this study suggesting that NDRG1 could be used as a marker for evaluation of efficiency of differentiation therapy. Differentiation therapy is based on the concept that differentiation-inducing agents can force cancer cells to arrest at an immature or poorly differentiated state and to resume the process of maturation [19]. It describes the enforced differentiation of primary tumors with therapeutic compounds [34]. We suggest NDRG1 as a marker to evaluate the efficiency of such drugs in vivo in animal models as well as cancer patients. In fact, NDRG1, together with p21 and p53, has been used for treatment evaluation in a Phase I clinical trial of the sequential combination of Irinotecan followed by Flavopiridol [38]. Furthermore, up-regulation of NDRG1 after incubation of cancer cells with phorbol esters and vitamin A and D has been reported [23]. All of these agents are considered as major candidates for differentiation therapy [39]. Moreover, based on data obtained from mouse in vivo model used in this study (Fig. 5) molecular approach to up-regulate NDRG1 expression in cancer tissue also could be considered as an anti-cancer strategy by inducing differentiation in breast cancer tissue.

In conclusion, considering the fact that NDRG1 is down-regulated in several malignancies (compared to normal tissues) and there is a direct relationship between NDRG1 expression and favorable prognosis in several types of cancers, understanding the mechanism(s) of action of NDRG1 regarding differentiation and the pathways controlling its expression as well as oncogenes suppressing its expression might provide valuable information for both cancer prevention and treatment.

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Conflict of Interest Statement The authors declare that they do not have any conflict of interest.

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