Introduction

Breast cancer neovascularization (BCN), involving both angiogenesis and vasculogenesis, is a key event in tumorigenesis, invasion and metastasis. In recent decades, significant progress has been made toward understanding the pathophysiologic mechanisms of BCN. However, the development of BCN is a complicated and dynamic process, involving multiple stimuli, multiple cytokines and multiple cell-type participation, and the specific mechanisms underlying its progression are largely unknown.

Hypoxia, a common feature of malignant tumors, can be detected in central regions of solid tumors as well as during embryonic development [1]. Hypoxia regulates many transcription factors, including hypoxia-inducible factor (HIF)-1α, which controls hypoxia-induced angiogenic factors such as vascular endothelial growth factor (VEGF) [2]. VEGF functions as a survival factor in endothelial cells and tumor cells via VEGF receptors that are also up-regulated by hypoxia [3]. Thus, increased VEGF expression by hypoxia, and thereby promotion of angiogenesis, may be a prerequisite for progressive growth and metastasis of solid tumors, such as breast cancer. The tumor suppressor genes p53 and VHL inhibit angiogenesis [4,5]. Mutation or loss of function of these suppressors results in high vascularization in malignant tumors. VHL and p53 could promote degradation of HIF-1α by ubiquitin-mediated proteolysis and suppress HIF-1α-stimulated transcription [6]. VEGF expression is negatively regulated by p53 and VHL and is frequently overexpressed along with HIF-1α in human cancers [4,7]. To understand the interactions between angiogenesis promoting factors and angiogenesis suppressing factors, it is necessary to develop treatment strategies that specially target BCN.

NDRG2, a member of the N-myc downstream-regulated gene family, belongs to the alpha/beta hydrolase superfamily. It was first cloned in our laboratory from a normal human brain cDNA library by subtractive hybridization (GenBank accession no. AF159092) and is regarded as a tumor suppressor gene (TSG) that is transcriptionally repressed by c-Myc [8,9,10]. Accumulated evidence indicates that NDRG2 is down-regulated or undetectable in many human cancers [11,12]. The role of
NDRG2 in breast cancer has also been studied. Recently, it has been shown that breast cancer cells have low or undetectable expression of NDRG2 compared with the high expression of NDRG2 in normal tissues [13]. Further studies have found that NDRG2 is able to inhibit proliferation and enhance apoptosis in many malignant tumors [14]. In addition, NDRG2 could induce BMP-4 expression and inhibit the metastatic potential of breast cancer cells, specifically via suppression of MMP-9 activity [15]. In our previous study, we have verified also that, as a regulator, NDRG2 could modulate the process of breast cancer cell adhesion and invasion through regulating CD24 expression [16]. However, the specific effect of NDRG2 on breast cancer angiogenesis is still unknown.

In this study, we first demonstrate that NDRG2 is a regulator participating in the progression of breast cancer angiogenesis. Using adenovirus, NDRG2 expression, we verified that Ad-NDRG2 could decrease VEGF secretion by MCF-7 cells, and this MCF-7 conditioned medium significantly inhibited HUVEC proliferation, tube formation and invasion. Furthermore, Ad-NDRG2 up-regulated the expressions of p53 and VHL while down-regulating the expressions of VEGF and HIF-1α. Ad-NDRG2 also inhibited tumor growth and angiogenesis in a nude mouse model. Our results present a novel role for NDRG2 in the regulation of breast cancer angiogenesis.

Results

NDRG2 expression in breast cancer cell lines

The tumor suppressor gene NDRG2 is down-regulated or undetectable in many human cancers. Here, NDRG2 expression in seven breast cancer cell lines was investigated by western blot and RT-PCR. The results showed that the expression of NDRG2 was higher in ZR-751 and T47D cell lines and lower in MDA-MB-231 and MCF-7 cell lines (Fig. 1A). To further determine the role of NDRG2, we chose to use MCF-7 cells (with wild-type p53) as our experimental model in the following studies.

NDRG2 up-regulation by Ad-NDRG2 in MCF-7 cells

To evaluate the up-regulation of NDRG2 expression, western blot and RT-PCR analyses were performed. As shown in Figure 1B, after infection by Ad-NDRG2, the expression of NDRG2 was successfully increased at both the protein and mRNA levels in MCF-7 cells, which express a low level of endogenous NDRG2.

Suppression of HUVEC proliferation by up-regulating NDRG2 in MCF-7 cells

To investigate the effect of NDRG2 up-regulation in MCF-7 cells on HUVEC proliferation, an MTT assay was designed. First, HUVECs grown in serum-free medium conditioned by MCF-7 cells showed an increase in the proliferation rate, as revealed by the culture time (Fig. 2A). Next, medium conditioned by Ad-NDRG2 infected MCF-7 cells markedly inhibited HUVEC proliferation, which correlated with the conditioning time of the medium (Fig. 2B). Notably, compared to normoxic conditions, these effects were much more significant under hypoxic conditions.

Alterations of HUVEC tube formation and invasion by regulating NDRG2 in MCF-7 cells

To test the effect of NDRG2 on angiogenesis, tube formation and invasion assays of HUVECs were performed on Matrigel under hypoxia. Ad-NDRG2 and NDRG2 siRNA (siNDRG2) were applied respectively to overexpress or knockdown NDRG2. As the control group, Ad-LacZ-MCF-7 or scramble-MCF-7 cells, HUVECs associated with one another and formed microtubes after 6 h. The medium from Ad-NDRG2-MCF-7 cells could repress the tube formation of HUVECs, but that from siNDRG2-MCF-7 cells promoted the tube formation of HUVECs (Fig. 3A and 3B). The invasion assay revealed that the medium from Ad-NDRG2-MCF-7 cells inhibited HUVECs’ invasion ability; however, that from siNDRG2-MCF-7 cells increased a lot compared with the Ad-LacZ-MCF-7 or scramble-MCF-7 cells respectively (Fig. 3C and 3D). These data raised the possibility that NDRG2 overexpression or knockdown in MCF-7 cells might mediate the production of extracellular matrix components that in turn are involved in the tube formation and invasion of HUVECs.

Regulation of the expression of hypoxia-induced, angiogenesis related factors by up-regulation of NDRG2 in MCF-7 cells

To further explore whether products of resulting from NDRG2 up-regulation in MCF-7 cells may be responsible for inhibition of HUVEC growth, we measured the VEGF level in cell culture medium under normoxic or hypoxic conditions. ELISA assay results showed that, compared to normoxic conditions, hypoxia could significantly increase VEGF secretion into the cell culture medium in a time-dependent manner (Fig. 4A). Furthermore, VEGF secretion was decreased in MCF-7 cells infected by Ad-

Figure 1. Expression of NDRG2 in breast cancer cell lines and the effect of Ad-NDRG2. (A) T-47D, MCF-7, BCAP-37, HBL-100, ZR-751, SK-BR-3 and MDA-MB-231 cells were collected for extraction of protein and RNA and analyzed for NDRG2 expression using western blot and RT-PCR. (B) NDRG2 low-expression MCF-7 breast cancer cells were infected by adenovirus carrying NDRG2 (Ad-NDRG2) or negative control LacZ (Ad-LacZ). Thereafter, protein and RNA from these cells were extracted and subjected to western blot and RT-PCR analysis. β-actin was used as a loading control. The data are representative of three independent experiments. doi:10.1371/journal.pone.0032368.g001

Mechanism of NDRG2 in CNV Angiogenesis
angiogenesis in vivo

in Figure 5A, the Ad-tumors (approximately 200 mm³) grown in nude mice. As shown or PBS every 3 days into pre-established, human MCF-7 breast transcription of HIF-1 regulating p53 and VHL expression or by directly controlling the results showed that HIF-1 mediated cell activities in breast cancer, western blot was performed to examine the expression levels of these factors. The data are the mean ± standard deviation (SD) for three independent experiments. Statistical significance was assessed using one-way ANOVA and Student’s t-test. * or ** indicates P<0.05 or P<0.01, respectively, when compared with the control. doi:10.1371/journal.pone.0032368.g002

NDRG2 in a time-dependent manner, especially under hypoxic conditions (Fig. 4B). To determine whether these hypoxia-induced, angiogenesis related factors are involved in NDRG2-mediated cell activities in breast cancer, western blot was performed to examine the expression levels of these factors. The results showed that HIF-1α expression was decreased in Ad-NDRG2-MCF-7 cells, and this was in accordance with the decreased VEGF secretion. In contrast to the effect on VEGF and HIF-1α, the expressions of p53 and VHL were up-regulated by Ad-NDRG2 under hypoxia (Fig. 4C). Thus, it is possible that NDRG2 influences HIF-1α and VEGF expression by indirectly regulating p53 and VHL expression or by directly controlling the transcription of HIF-1α and VEGF.

Suppression of tumor growth and angiogenesis in a nude mouse model by intratumoral injection of Ad-NDRG2

To investigate the effects of Ad-NDRG2 on tumor growth and angiogenesis in vivo, we injected 1 × 10⁷ PFU Ad-NDRG2, Ad-LacZ or PBS every 3 days into pre-established, human MCF-7 breast tumors (approximately 200 mm³) grown in nude mice. As shown in Figure 5A, the Ad-NDRG2 group achieved a sustained and significant arrest of tumor growth (71% decrease in mean tumor volume on day 21 compared with the control group), whereas the growth of tumors injected with Ad-LacZ was not significantly inhibited (3.4% decrease compared with the control group). The mice were sacrificed at 21 days after beginning intratumoral injections, and the tumors were removed for analysis of the vascularization. Immunolabeling for VEGF and HIF-1α were lower in tumors excised from mice in the Ad-NDRG2 group. Moreover, the p53 and VHL expression levels in the Ad-NDRG2 group were dramatically increased compared to the control group. The microvessel density (MVD) of the tumors was examined after immunolabeling for CD31, a typical vascular endothelial cell marker. In the Ad-NDRG2 group, the MVD of the tumors was inhibited compared with the control and Ad-LacZ groups. There was no significant difference between the control and Ad-LacZ groups (Fig. 5B and 5C).

Discussion

Although surgical resection, adjuvant and endocrine therapy are commonly used to treat breast cancer patients, the overall survival rate for breast cancer requires improvement. Therefore, the development of biological therapies for breast cancer is urgently required. Recent reports have shown, in breast cancer patients, that NDRG2 expression is significantly reduced compared to breast normal tissue [13]. Further studies have found that NDRG2 inhibits the proliferation, adhesion and invasion of breast cancer cells [14,16]. In addition, our previous study found that NDRG2 could inhibit the metastatic potential of breast cancer cells, specifically via suppression of CD24 or MMP-9 expression [16]. Interestingly, silencing NDRG2 attenuates p33-mediated apoptosis, whereas overexpression of NDRG2 suppresses tumor cell growth regardless of the presence or absence of p53 [14]. Therefore, NDRG2 is required for p33-mediated apoptosis but not for proliferation. These data indicate that an association exists between NDRG2 expression and reduced breast cancer malignant behavior and that NDRG2 may be a novel target for future biological therapies for breast cancer. However, to date, the roles of NDRG2 in breast cancer angiogenesis have not been explored. In this paper, our data revealed that NDRG2 overexpression in breast cancer cells could suppress HUVEC proliferation, tube formation and invasion by reducing VEGF and HIF-1α expression and increasing p53 and VHL expression. Importantly, tumor growth and angiogenesis in a nude mouse model were inhibited by intratumoral injection of Ad-NDRG2.

Recently, several reports have shown that NDRG2 is a target gene of HIF-1α, and hypoxia treatment dramatically up-regulated NDRG2 expression in many human cancer cell lines [17,18]. Knockdown of HIF-1α expression did not change NDRG2 expression but could abolish hypoxia-induced up-regulation of NDRG2. In our study, infection by Ad-NDRG2 reduced HIF-1α expression in a time-dependent manner in MCF-7 cells. Based on these results, we believe that NDRG2 is a hypoxia-regulated gene, NDRG2 and HIF-1α could regulate each other, and there might be feedback loops between them. Furthermore, our data showed that VEGF secretion was decreased in MCF-7 cells infected by Ad-NDRG2 in a time-dependent manner, especially under hypoxic conditions. This suggests that NDRG2 may be involved in hypoxia-induced angiogenesis.

Loss of wild type p53 and VHL could promote angiogenesis by relieving inhibition of VEGF production. Several findings demonstrated that p53 and VHL could inhibit VEGF expression by regulating the transcriptional activity of Sp1 and by down-regulating Src kinase activity under hypoxia [19,20]. Moreover, both p53 and VHL can promote the degradation of HIF-1α to indirectly suppress VEGF expression [21,22,23]. There is evidence
indicating that the expression of VEGF is negatively regulated by p53 and VHL and is frequently overexpressed along with HIF-1α in human cancers.

The present study has shown that NDRG2 overexpression in breast cancer cells can inhibit the proliferation, tube formation and invasion of HUVECs, preventing the participation of these cells in hypoxia-induced, tumor angiogenesis. This effect may be related to the inhibition of VEGF secretion. Both in vitro and in vivo up-regulation of NDRG2 by Ad-NDRG2 increased p53 and VHL expression and decreased VEGF expression. It is possible that the down-regulation of VEGF by NDRG2 overexpression might be the consequence of the up-regulation of the tumor suppressor genes p53 and VHL. In summary, our results provide the first evidence that NDRG2 contributes to breast cancer angiogenesis by controlling the expression of angiogenesis related factors. Further investigation into the molecular mechanism of NDRG2 action may offer a novel approach for treating breast cancer, specifically by targeting breast cancer angiogenesis.

Figure 3. Effect of MCF-7 preconditioned media on HUVEC tube formation and invasion. (A and B) The tube formation assay was performed as described in Material and Methods. HUVECs were plated on Matrigel and incubated under hypoxia for 6 h. Tube lengths were measured with Image-Pro Plus software; the histograms represent the quantification of the tube length of HUVECs. (C and D) The invasion assay was performed as described in Material and Methods. After 4 h under hypoxia, the amount of invaded cells was calculated in five random high-power fields (400 ×), and the number from the control group was used as a control. The histogram represents the quantification of cells that invaded. The data are the mean ± standard deviation (SD) for three independent experiments. Statistical significance was assessed using one-way ANOVA and Student’s t-test. * or ** indicates P<0.05 or P<0.01, respectively, when compared with the control. The Ad-NDRG2 panel of Figure 3A is excluded from this article’s CC-BY license. See the accompanying retraction notice for more information. doi:10.1371/journal.pone.0032368.g003
Materials and Methods

Cell lines and reagents

Human breast cancer cell lines T-47D, MCF-7, BCAP-37, HBL-100, ZR-751, SK-BR-5 and MDA-MB-231, were obtained from the American Type Culture Collection (ATCC, USA) and cultured according to their directions and guidelines. Human umbilical vein endothelial cells (HUVECs) were obtained and cultured as described previously [21,22]. HUVECs were suspended in HuMedia-EG2 medium (Kurabo Industries) and then plated on plastic culture dishes. HuMedia-EG2 medium consisted of the base medium (HuMedia-EB2) supplemented with 2% fetal bovine serum, 10 ng/ml hEGF, 5 ng/ml hFGF-B, 1 mg/ml hydrocortisone, 50 μg/ml gentamicin, 50 μg/ml amphotericin B, and 10 μg/mL heparin. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO2. Before experiments, cells were cultured under serum-free conditions in M199 medium for 18 to 24 h. For hypoxia treatment, cells were cultured under hypoxic conditions (1% O2, 5% CO2 and 94% N2) and harvested at different times.

Figure 4. Effect of Ad-NDRG2 on the expression of VEGF, HIF-1α, p53, and VHL under normoxia or hypoxia. (A) MCF-7 cells were incubated for the indicated time (0, 6, 12, 24, 36 h) in normoxic (21% O2) or hypoxic (1% O2) conditions. After incubation, the media were analyzed for VEGF levels by ELISA assay, and the cell number was counted. The data are the mean ± standard deviation (SD) for three independent experiments. Statistical significance was assessed with one-way ANOVA and Student’s t-test. * or ** indicates P<0.05 or P<0.01, respectively, when compared with the normoxia or Ad-LacZ-MCF-7 group. N, normoxia; H, hypoxia. (C) MCF-7, Ad-LacZ-MCF-7 and Ad-NDRG2-MCF-7 cells were incubated under hypoxia for 0, 12, or 24 h. After incubation, the cells were analyzed by western blot assay. β-actin protein levels were used as a loading control.

Gene infection

A multiplicity of infection (MOI) of 40 was determined experimentally for MCF-7 cells. Cells were seeded in 6-well plates at a density of 5x10⁵ cells/well and incubated to reach approximately 80% confluence. After removing the medium, adenovirus expressing NDRG2 (Ad-NDRG2) or the negative control gene LacZ (Ad-LacZ) was added in serum-free DMEM, incubated for 2 h, replaced with fresh DMEM supplemented with 10% FBS and incubated for 48 h.

Gene transfection

MCF-7 cells were seeded in 6-well plates at a density of 5x10⁵ cells/well and incubated to reach approximately 80% confluence. The gene transfection assay was performed with the following constructs using Lipofectamine 2000 (Invitrogen, Carlsbad, Calif) according to the manufacturer’s instructions: a small interfering RNA (siRNA) that targeted NDRG2 (siNDRG2: forward, GCU-CUCUGGAAAUCUCUGAGGUUA; reverse, UUAAGAGCUAUAUUCUGCAGGAUGU) and its scramble siRNA (scramble: forward, UUCUCGGAACGUGCUGACAGTT; reverse, AGGUGACUCUGAGAAATGTT; Biomics Inc., Agoura Hills, Calif). Cells were exposed to siRNA in DMEM for 6 h, after
which the medium was replaced with DMEM containing 10% FBS and the cells were incubated for 48 h.

RT-PCR
RNA extracted from cells with Trizol reagent (Invitrogen, Life Technologies, USA) was converted to cDNA using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, CA). Total RNA (5 µg) was then reverse transcribed into cDNA using an AMV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer’s instructions. The cDNA was subjected to PCR amplification using the following primers: NDRG2, 5’-ATGGCCCGAGCTGCGAGAGGTGC-3’ (sense) and 5’-TGAAGAAGGAGGCTCATGGGTTGG-3’ (antisense); b-actin, 5’-GATCATTCGCTCTCCCTTAGC-3’ (sense) and 5’-TGTTGGACCTGGGAGGACT-3’ (antisense). The PCR mixture (25 µl) containing a Taq polymerase from Promega was denatured at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 10 min. The PCR products were separated in 3% agarose gels by electrophoresis and visualized with ethidium bromide under a UV light.

Western blot
MCF-7 cells were homogenized in RIPA lysis buffer and insoluble material was removed by centrifugation at 4°C. From each sample, 80–100 µg of total protein extract was resolved by 12% SDS-PAGE, and transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). The membranes were blocked in 5% milk and probed with the antibodies overnight at 4°C. Membranes were washed and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotech) for 1 h at 37°C. Chemiluminescent HRP substrate solution (Millipore, USA) was used to develop the images.

MTT assay
HUVECs were plated at a density of 4×10⁴ cells/cm² in 24-well plates in complete medium and allowed to adhere overnight. The conditioned culture medium from MCF-7 cells was placed in the wells, and the whole plate was incubated in a hypoxic chamber. HUVEC proliferation was measured using a modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. After the inserts were removed, 100 µl MTT was added per well, and HUVECs were incubated for 4 h. The formazan crystals formed were dissolved in 750 µl dimethyl sulfoxide after the medium was aspirated. The solution was transferred to a 96-well plate and the optical density value was recorded at 570 nm on a Microplate Reader (Model 680, Bio-Rad, USA). The results were expressed relative to the OD value of HUVEC monocultures on day 1 of the assay.

ELISA assay
To assess VEGF secretion in the supernatants of infected cells, the cells were incubated in serum-free medium under normoxia or hypoxia. After 0–36 h, the media were collected, centrifuged to remove cellular debris, and stored at −70°C until assayed for VEGF. VEGF secreted in the culture medium was measured by ELISA according to the manufacturer’s instructions. Data were expressed in pg VEGF/10⁵ cells/ml.

Tube formation assay
Sterile 24-well plates were coated with 200 µl Matrigel and incubated at 37°C for 1 h to form gels. After polymerization of the gels, 1.0×10⁵ HUVECs were seeded into each well and incubated with 1.0 ml DMEM containing 1% FBS. Next, media from MCF-7 cells were placed in the wells under hypoxia for 6 h. Five different fields were randomly chosen in each well, and photographs were taken. The length of the tubes was measured using Image-Pro Plus software (Media Cybernetics, L.P., Silver Spring, MD, USA) and was expressed as total length (mm) per microscopic field for each well.

Invasion assay
The HUVECs migration assay was performed using Matrigel-coated Costar Transwell inserts with an 8 µm pore-size. Briefly, 5.0×10⁴ HUVECs were seeded onto inserts and incubated with M199 medium containing 1% FBS. After a 1 h attachment, the inserts were transferred to 24-well plates containing media from Ad-LacZ-MCF-7 or Ad-NDRG2-MCF-7 cells. After incubation for 4 h under hypoxic condition, the inserts were fixed with 4% paraformaldehyde and stained with hematoxylin and eosin. The number of invaded cells was counted using phase contrast microscopy (400×). Five randomly chosen fields were counted per insert.

Xenograft study in nude mice
For inoculation into nude mice, MCF-7 cells were washed with PBS, digested with trypsin, and resuspended in serum-free DMEM medium. After centrifugation (800 rpm), cell pellets were resuspended in DMEM. The cell suspension (5×10⁶ cells in a volume of 100 µl PBS) was injected subcutaneously into the hind legs of 4-week-old female BALB/C athymic (nu/nu) mice (SLAC Laboratory Animal Company, Shanghai, China) [24]. When tumors reached a volume of 200 mm³, the mice were arbitrarily assigned to different groups (n = 6 each) to receive intratumoral injections of 10⁵ PFU Ad-NDRG2, Ad-LacZ, or PBS. Intratumoral injections were repeated every 3 days for a total of 20 days. Tumors were measured (perpendicular diameters) every day and their volumes calculated. On day 20, the mice were sacrificed and their tumors removed for analysis. Tumor volumes were calculated based on caliper measurements of the length and width of the lesions using the following formula: 0.5 × length × width². The growth curve was then derived from these data.
All the experimental procedures were conducted in accordance with the Detailed Rules for the Administration of Animal Experiments for Medical Research Purposes issued by the
Ministry of Health of China and received ethical approval by the Animal Experiment Administration Committee of the Fourth Military Medical University (Xi’an, P. R. China). All efforts were made to minimize the animals’ suffering and to reduce the number of animals used.

Immunohistochemistry

Immunohistochemical staining was performed to assess the protein expression of VEGF, HIF-1α, VHL, p53 and CD31, as described previously [25]. For immunohistochemistry, formalin-fixed tumor tissues were embedded in paraffin, and serial 4 μm sections were obtained using a Leica microtome. For staining, tumor sections were dewaxed in toluene, rehydrated in an alcohol gradient, permeabilized in citrate buffer (pH 6.0), quenched with 3% H2O2 for 5 min to eliminate endogenous peroxidase activity, washed in PBS, incubated overnight with different antibodies and then with biotinylated goat anti-rat or anti-rabbit IgG antibody for 15 min. After washing, sections were incubated with streptavidin–peroxidase, lightly counterstained with hematoxylin, and observed under a photomicroscope.

Statistical analysis

SPSS 13.0 software was used to perform statistical analyses. Data are presented as the mean ± SD, and statistical comparisons between groups were made using one-way ANOVA followed by Student’s t-test. P<0.05 was considered statistically significant.

Author Contributions

Conceived and designed the experiments: JM. Performed the experiments: XY QW LW. Analyzed the data: YX SL LM LY. Contributed reagents/materials/analysis tools: QZ HR YC. Wrote the paper: JM. Critical reading and design, obtained funding, critical revision of the manuscript for important intellectual content and study supervision: JZ.

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