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Overexpression of ΔNp63α induces a stem cell phenotype in MCF7 breast carcinoma cell line through the Notch pathway

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To elucidate a role of ΔNp63α in breast cancer, the expression levels of p63, estrogen receptor, progesterone receptor, p53, CK5, cerBb-2, and Notch1 were assayed in 50 clinical breast cancer specimens using immunochemistry. P63 was highly expressed in a subset of breast cancer with basal-like features. We then transfected MCF7 cells with ΔNp63α plasmid, and assayed its cancer stem cell-like features after transfection. Overexpression of ΔNp63α in MCF7 cells increased the percentage of $CD24^{-}CD44^{+}$ subpopulation from 2.2 ± 0.2% to 25.1 ± 1.5% ($P < 0.05$) and led to increased cancer cell proliferation, clonogenicity, anchorage-independent growth, and the incidence of xenograft grown *in vivo*. In addition, ΔNp63α overexpressing cancer cells were more drug resistant. Further studies suggested ΔNp63α-induced activation of the Notch pathway may play a role in these effects. Chromatin immunoprecipitation confirmed that ΔNp63α could directly bind to Notch1. In clinical breast cancer specimens, the expression level of p63 was also found to positively correlate with the expression level of Notch1. Our results suggest that ΔNp63α might serve as a tumor initiating transcription factor in breast cancer. (*Cancer Sci* 2010; 101: 2417–2424)

Breast cancer is the most common type of cancer in women. Despite significant advances in diagnosing and treating breast cancer, a large number of patients will relapse and die of metastasis. Breast cancer stem cells are thought to be the root of breast cancer and failure to eradicate cancer stem cells may be the main reason for relapse.[1,2] The hypothesis that breast cancer is a stem cell disease entails the notion that breast cancer may result from transformation of normal stem and/or progenitor cells. Breast cancer stem cells have been prospectively identified as $CD24^{-}CD44^{+}$.[3] Like their normal counterparts, cancer stem cells undergo the process of self-renewal and differentiation. The growth of the tumor mass relies on the proliferation and self-renewal capacity of these rare cancer stem cells. Therefore, a clearer understanding of the genetic programs underlying cancer stem cell self-renewal may lead to identification of new therapeutic targets, which will have important implications for prevention and clinical management of breast cancer.

P63, a homolog of the p53 tumor suppressor gene, has two types of protein isoforms (TA, transactivating and ΔN, non-transactivating) due to alternative promoter usage. Each type yields three isotypes (α, β, and γ) because of differential splicing at its C-terminus.[4] Thus there are six p63 isoforms with a complex array of similarities and differences in their structural domains and cellular functions. P63 plays an essential role in epithelial development because p63-null mice have a near complete absence of the epidermis, mammary, and prostate tissue and a profound failure in limb formation.[5,6] ΔNp63 lacks the N-terminal transactivation domain and was originally thought to function as a ''dominant negative'' protein that blocks the function of the corresponding full-length proteins. However, it was later discovered that ΔNp63 isotypes might function as transcription factors.[7,8] In adult breast tissue, ΔNp63 expression is restricted to the basal myoepithelial cell layer, which is known to contribute to proliferation, differentiation, and polarity of mammary epithelia, and is thought to contain breast stem cells.[9,10] Deugnier *et al.*[11] reported that mammary stem cells were ΔNp63 positive and could generate identical daughter cells that retained their *in vivo* developmental potential. ΔNp63α is the main isotype expressed in both normal and malignant breast tissue.[12,13] Furthermore, ΔNp63α is markedly expressed in basal-type breast cancer, an uncommon type with stem cell-like features. The importance of ΔNp63α in mammary stem cells and its expression in basal-like breast cancer suggest it might be an important regulator of breast cancer stem cells. Despite all of these findings, a definite role for ΔNp63α in breast cancer development is still lacking. Some studies showed ΔNp63 could act as an oncogene to promote proliferation and inhibit apoptosis, whereas other studies showed ΔNp63 could cause cell cycle arrest and apoptosis.[7] Recently, ΔNp63 has been shown to act as a tumor suppressor in breast cancer.[14]

We hypothesized that ΔNp63α might play a critical role in self-renewal of breast cancer stem cells. In this study we found that ΔNp63 was mainly expressed in a subpopulation of breast cancer patients that were estrogen receptor (ER), progesterone receptor (PR) and cerBb-2 negative (triple negative). Then we used the MCF7 breast cancer cell line as a model to study the role of ΔNp63α. We provide evidence that overexpression of ΔNp63α increases the fraction of cancer stem cell-like cells in MCF7 cells through the Notch pathway.

Materials and Methods

Breast cancer specimens and immunohistochemistry. A total of 50 clinical breast cancer specimens and the corresponding clinicopathologic data were obtained from the Institute of Pathology, Southwest Hospital, Third Military Medical University (Chongqing, China). All patients gave informed consent on the use of tumor specimens for research purposes. Immunohistochemical procedures were carried out as previously described.[15] Results of immunohistochemical staining were categorized independently by two experienced pathologists, according to the number of immunopositive cells, in a blinded fashion. Tumors were considered to be positive for p63 and CK5 when any neoplastic cell displayed distinct brown nuclear or cytoplastic staining, respectively.[15] The cases were

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interpreted as ER^+, PR^+, or $P53^+$ if more than 10% of the neoplastic cells showed nuclear staining.[16,17] The intensities of immunohistochemical staining with Notch1 and cerBb-2 were defined as follows: negative, the reaction was indistinguishable from the background or <5% of tumor cells were stained; low, 5–30% of tumor cells were positively stained; and high, >30% of tumor cells were positively stained. This study was approved by the institutional review boards and ethics committees of both Southwest Hospital and Peking Union Medical College.

Cell culture and plasmid construction. The human breast carcinoma cell lines MCF7, MDAMB453, MDAMB157, and SKBR3 were obtained from the Cell Center of Peking Union Medical College and were cultured in desired medium. PCDNA3.1 ΔNp63α plasmid was a kind gift from Dr Kangjian Wu (Mayo Clinic, Rochester, MN, USA) and was confirmed by direct sequencing. MCF7 cells were either transfected with PCDNA3.1 ΔNp63α plasmid or PCDNA3.1 plasmid. Lipofectin 2000 (Invitrogen, Carlsbad, CA, USA) was used for cell transfection. Stable colonies were selected by G418 resistance.

MTT assay. Proliferation of cells stably transfected with ΔNp63α (hereafter referred to as ΔNp63α cells) was compared to control cells transfected with the empty vector (hereafter referred to as empty vector cells), using the MTT method, as we described previously.[18]

Mammosphere culture and soft agar assays. Single cells were suspended at 500 cells per mL in a serum-free DMEM-F12 (Invitrogen) with 10 ng/mL basic fibroblast growth factor, 20 ng/mL epidermal growth factor, 5 ug/mL insulin, and 0.4% BSA (Sigma, St. Louis, MO, USA). Cell suspension (2 mL) was added into each well in super-low 6-well plates (American Coroning Company, Corning, NY, USA) and grown at 37°C with 5% CO_2. Cells were allowed to grow in anchorage independence for 7 days and the numbers of spheres were counted. Mammosphere forming efficiency was calculated by dividing the number of mammospheres (≥60 μm) formed by the original number of single cells seeded and expressed as a percentage. Soft agar assay were carried out as previously described.[19] In some experiments, ΔNp63α overexpressing MCF7 cells were cultured in the presence of GSI-1 (5 μM; Merck, Darmstadt, Germany) or 0.001% DMSO (vehicle control) for 48 h before they were assayed for colony formation in soft agar.

Flow cytometry. Subconfluent cells were trypsinized, washed in PBS with 1% BSA and adjusted to 2×10^6 cells/mL. A total of 1×10^6 cells were incubated with antibodies for 30 min at room temperature. Unbound antibody was washed off and cells were analyzed within 1 h post-staining on a BD FACSCalibur (BD Biosciences, San Jose, CA, USA). The following antibodies were used: CD24; CD44 and rat IgG; FITC-conjugated antibody (BD Pharmingen, San Jose, CA, USA); Notch1; Notch2; Jagged1; and Jagged2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The primary and secondary antibodies were used at a concentration of 1:300 and 1:1000, respectively.

For determination of cell apoptosis, 60–70% confluent ΔNp63α cells and empty vector cells were treated with 2 μM adriamycin in fresh culture medium. After 24 h, cells were collected and washed twice with pre-chilled PBS. The cell concentration was adjusted to $5–6 \times 10^5$ cells/500 μL with pre-chilled binding buffer and incubated with annexin V–FITC and propidium iodide (PI) for 10 min in the dark on ice. Then 400 μL pre-chilled binding buffer was added. The samples were then assayed on the BD FACSCalibur.

Tumorigenicity assays. Six-week-old female NOD/SCID mice were anesthetized with ketamine/xilazine. Cells were then injected into the fourth mammary gland of the right side. The animals were killed after 28 days of injection, and a biopsy was carried out to determine the tumor growth. The percentage of tumor was calculated as the number of mice with tumor/total number of mice injected. This study was approved by the institutional review boards and ethics committees of Peking Union Medical College.

Double immunofluorescence staining. The mammospheres were collected by gentle centrifugation (800*g*, 10 sec), embedded in Matrigel and solidified at 37°C for 30 min. Next they were fixed in 10% paraformaldehyde and embedded in paraffin. For double immunofluorescence staining, the deparaffined sections were microwave heated in citrate buffer for 5 min, washed in PBS, blocked with 10% horse serum, and incubated with CD24 and CD44 antibody (BD Pharmingen) at room temperature for 1 h. The slides were then washed in PBS and counterstained with 5 μg/mL Hoechst 33342 (Sigma).

Real-time PCR. Real-time PCR was carried out as previously described.[18] Amplification of targeted genes was achieved using the primers in Table 1.

Western blot analysis. Protein lysates were prepared using Radio Immunoprecipitation Assay cell lysis buffer. Protein concentrations were determined using a Bradford dye-based assay (Beyotime, Haimen, China). Total protein (25 μg) was subjected to SDS-PAGE followed by immunoblotting with appropriate antibodies at the recommended dilutions. The blots were then incubated with peroxidase-linked secondary antibodies followed by enhanced chemical luminescent detection. Primary antibodies were anti-p63, anti-Notch1 (both Santa Cruz Biotechnology), and anti-notch-1 intracellular domain (NICD) (Cell Signaling Technology, Beverly, MA, USA). All secondary antibodies were purchased from NeoBioScience (Beijing, China).

Chromatin immunoprecipitation assays. We used a Chromatin Immunoprecipitation Kit (Millipore, Temucula, CA, USA) to carry out ChIP assays according to the manufacturer's instructions, and as described previously.[20] Primer sequences were 5′-GCTTGCATCAATTTCACTC-3′ and 5′-CAAACCCACAACCACAAA-3′.

Statistical analysis. The correlation between expression of p63 in tumor specimens and that of ER, PR, p53, CK5, cerBb-2, and Notch1 was analyzed using the χ^2-test. The statistical difference in the incidence of tumorigenicity between the cell types in the mouse xenograft model was assessed using Fisher's exact test. For means and standard deviations, a paired two-sided Student's *t*-test was used. *P*-values of 0.05 or less were considered to indicate statistically significant differences. SPSS version 12.0 (SPSS, Chicago, IL, USA) software was used for calculations.

Table 1. Nucleotide sequences of the primers used in RT-PCR and real-time RT-PCR

Gene	Forward	Reverse	Length (bp)
Np63	GGAAAACAATGCCCAGACTC	GTGGAATACGTCCAGGTGGC	294
Tp63	AAGATGGTGCGACAAACAAG	AGAGAGCATCGAAGGTGGAG	234
Notch1	CGCTGACGGAGTACAAGTG	GGTAGGAGCCGACCTCGTT	246
Notch2	GCCTTCGCCTCCTGTACTC	GCCCATTTAGGGGGTTGGT	166
Jagged1	CTCGGGTCAGTTCGAGTTGG	AGGCACACTTTGAAGTATGTGTC	144
Jagged2	ACTGGGACAACGATACCACC	AGTGGCGCTGTAGTAGTTCTC	170
β-actin	GCTCCTCCTGAGCGCAAGTA	GATGGAGGGGCCGGACT	117

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Results

P63 is mainly expressed in basal-like breast cancer. We first assayed p63 expression and its correlation with ER, PR, cerBb-2, CK5, and p53 in 50 clinical breast cancer samples by immunohistochemical staining using specific antibodies. There were eight $p63^+$ samples. They were all negative for ER and PR, and only one was cerBb-2^+. As shown in Table 2, p63 expression was mainly confined to ER^-, PR^-, cerBb-2^-, and $CK5^+$ samples. Most of these patients were basal-like breast cancer.[17,21]

Table 2. Relationship between p63 expression and immunohistochemical features

Feature	$p63^+$	$p63^-$	*P*-value
Total	8	42	NA
Estrogen receptor			
Negative	8	16	0.001
Positive	0	26	NA
Progesterone receptor			
Negative	8	23	0.018
Positive	0	19	NA
p53 mutation			
Negative	0	27	0.001
Positive	8	15	NA
CK5			
Negative	0	29	0.093
Positive	8	13	NA
cerBb-2			
Negative	7	10	0.020
Low positive	1	17	NA
High positive	0	15	NA

P-values were calculated using the χ^2-test and Fisher's exact test. *P*-values were considered to be statistically significant at $P < 0.05$ (two-sided). NA, not applicable.

Overexpression of ΔNp63α increases the percentage of breast cancer stem cell-like cells in MCF7 cells. As p63 contains various isotypes, we used quantitative real-time RT-PCR (qRT-PCR) to assay which isotypes were more highly expressed in MCF7 cells. Consistent with a previous report,[13] the expression level of ΔNp63 RNA was 10-fold higher than TAp63 (data not shown). As ΔNp63α is the main isotype expressed in breast cancer,[13] we next overexpressed ΔNp63α in MCF7 cells to study its role in breast cancer cells. Stable overexpression colonies were selected through G418 (500 μg/mL) resistance. ΔNp63α overexpressing cells and empty vector control cells were cultured in the same conditions. Quantitative RT-PCR and Western blot analysis showed that the ΔNp63α mRNA level was eightfold greater than its endogenous isoform (Fig. 1A,B). ΔNp63α overexpression had no significant effect on cell morphology (data not shown).

A previous report showed that breast cancer stem cells were $CD24^-CD44^+$.[3] However, there were only a few $CD24^-CD44^+$ cells in MCF7 cells. Overexpression of ΔNp63α increased the percentage of $CD24^-CD44^+$ subpopulation from 2.2 ± 0.2% to 25.1 ± 1.5% ($P < 0.001$, Fig 1C).

We assessed their colony formation ability in soft agar. As shown in Figure 2(A), the number of colonies grown in soft agar were increased by nearly sixfold in MCF7 cell clones transfected with ΔNp63α compared to empty vector cells or parental cells. When one thousand of ΔNp63α overexpressing cells were seeded in soft agar they formed an average of 239 colonies, compared to 37 and 39 for parental and empty vector cells, respectively. The colony formation efficiencies were 23.9 ± 5.1%, 3.6 ± 0.2%, and 3.7 ± 0.2% for the ΔNp63α, parental, and empty vector groups, respectively ($P < 0.001$, Fig. 2B).

Breast cancer stem cells can grow into mammospheres in super-low adherence plates in conditioned media. We assayed the mammosphere forming efficiency of empty vector cells and ΔNp63α cells. As expected, ΔNp63α cells had a higher mammosphere forming efficiency than empty vector cells. Approximately 41 mammospheres were formed per 1000 ΔNp63α cells (mammosphere forming efficiency 4.1 ± 0.5%), whereas

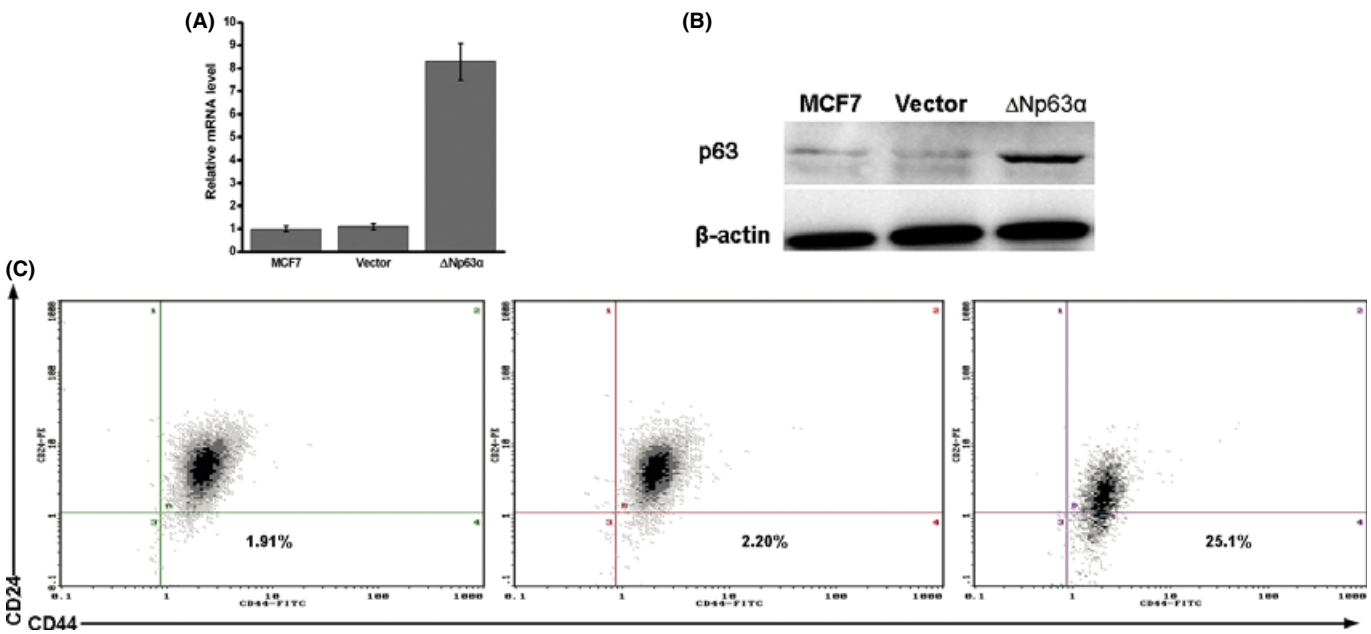

Fig. 1. Expression of ΔNp63α in parental, empty vector, and ΔNp63α-transfected MCF7 breast carcinoma cells. (A) Quantitative RT-PCR assay for p63. (B) Western blot analysis for p63. (C) Cells were stained with antibodies against CD44 and CD24, and detected by FACS. The frequency of $CD24^-CD44^+$ as a percentage of cancer stem cells are shown in the dot plots.

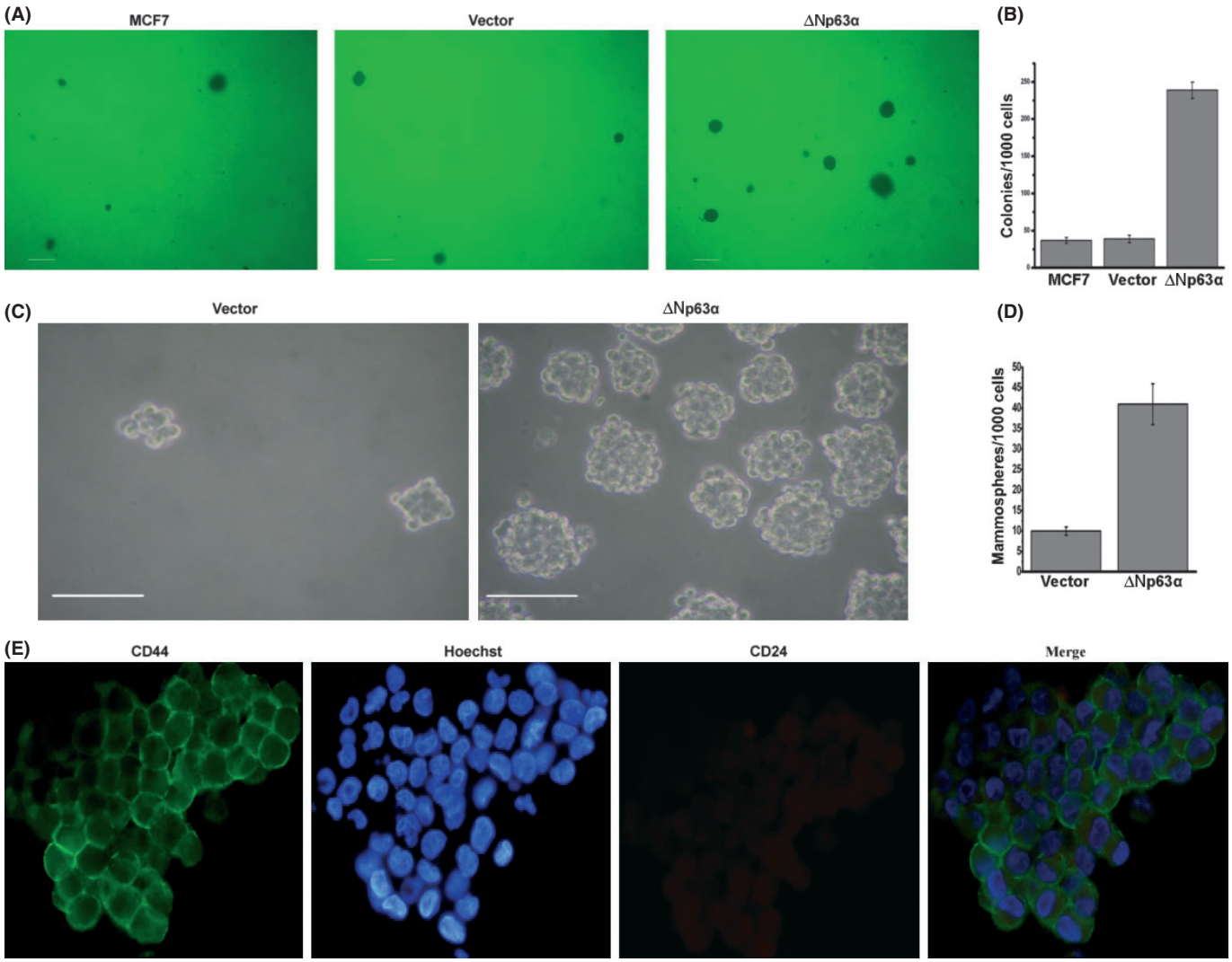

Fig. 2. One thousand of parental MCF-7 breast carcinoma cells, empty vector, and MCF-7 cells overexpressing ΔNp63α were cultured in 0.3% agar plates and stained by crystal violet (A, bar = 200 μm). Representative results of three independent experiments are shown (B). Representative pictures of mammosphere forming efficiency of empty vector and ΔNp63α cells (C, bar = 100 μm) and statistical results of three independent experiments (D). (E) Double immunofluorescence staining of mammospheres by CD24 (red) and CD44 (green). Nuclei were counterstained with Hoechst 33342 (blue). Magnification, ×400.

approximately 10 mammospheres were formed per 1000 empty vector cells (mammosphere forming efficiency 1.0 ± 0.1%; $P < 0.05$, Fig. 2C,D). Phenotype analysis showed that the mammospheres were mainly composed of $CD24^{-}CD44^{+}$ cells (Fig. 2E).

High tumorigenicity *in vivo* is one of the hallmarks of cancer stem cells, therefore we used the tumorigenicity assay in NOD/SCID mice to study whether ΔNp63α overexpression promoted tumorigenicity *in vivo*. Cells were injected into the right side of the fourth mammary fat pad of NOD/SCID mice as a 10-fold dilution series from 5×10^5 to 5×10^3 cells per mouse. The ΔNp63α cells showed significantly higher tumorigenicity than empty vector cells (Table 3).

Cells were treated with adriamycin of various concentrations for 48 h. As shown in Figure 3(A), ΔNp63α overexpressing cells were more drug resistant than parental or empty vector cells. IC_{50} values of ΔNp63α, MCF7, and empty vector cells were 4.0, 0.5, and 0.5 μM, respectively.

To quantitate apoptotic cell death, we assayed annexin V/PI staining by FACS analysis. This assay detects both early apoptotic (annexin V^+, PI^-) and late apoptotic (annexin V^+, PI^+) cells.

Table 3. Tumorigenicity assay of MCF7 breast carcinoma cells overexpressing Np63 in NOD/SCID mice

	No. of cells injected		
	5×10^5	5×10^4	5×10^3
Np63	8/8	9/10	4/7
Vector	6/8	3/10	0/7
P-value	0.467	0.020	0.070

We treated 60–70% confluent cells with 2 μM adriamycin in fresh culture medium for 24 h. The early apoptotic cells were 13.8 ± 1.5% and 6.4 ± 0.9% for empty vector and ΔNp63α cells, respectively ($P < 0.001$). The late apoptotic cells were 3.5 ± 0.5% and 3.8 ± 0.6% for empty vector and ΔNp63α cells, respectively ($P > 0.05$, Fig. 3B).

ΔNp63α activates Notch pathway. Previous experiments have shown that p63 could directly activate Notch in keratinocytes.[22,23] If activated, the Notch pathway could promote mam-

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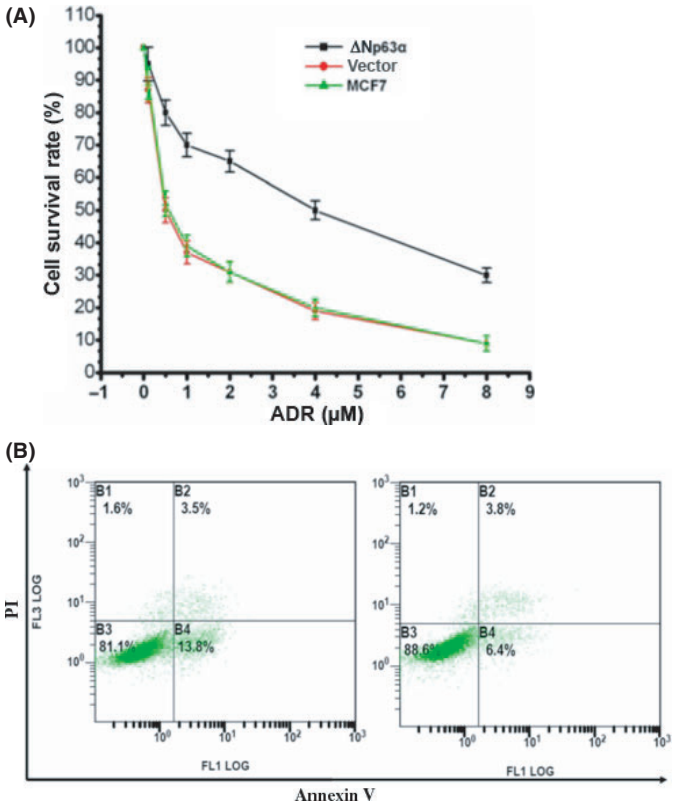

Fig. 3. Overexpression of ΔNp63α in MCF7 breast carcinoma cells significantly elevates drug resistant to adriamycin (ADM). (A) Cell viabilities were measured by MTT assay. (B) Annexin V/propidium iodide (PI) staining after treatment with 2 μM ADR for 24 h. Drug-induced apoptosis was indicated by annexin V and PI staining.

mosphere formation.[24] We hypothesized that overexpression of p63 might activate Notch in MCF7 cells, which enriched breast cancer stem cell-like cells. Using qRT-PCR, we found that the Notch1 transcript level in ΔNp63α cells was 3.8-fold higher than in empty vector cells. Notch2, Jagged1, and Jagged2 were two-fold higher (Fig. 4A). Flow cytometry analysis indicated that Notch1 and Notch2 expression were significantly higher in ΔNp63α cells than empty vector cells (0.85 ± 0.11 *vs* 0.50 ± 0.11 for Notch1; 0.84 ± 0.12 *vs* 0.54 ± 0.15 for Notch2, respectively; $P < 0.05$, Fig. 4B,C). But the fluorescence intensity of Jagged1 and Jagged2 was similar between ΔNp63α cells and empty vector cells (0.71 ± 0.10 *vs* 0.54 ± 0.15 for Jagged1; 0.71 ± 0.12 *vs* 0.54 ± 0.11 for Jagged2, respectively; $P > 0.05$, Fig. 4B,C). We also found that Notch1 intracellular domain was accumulated in ΔNp63α overexpression cells (Fig. 4D). We then tested whether Notch inhibitor GSI-1 could reverse ΔNp63α colony forming efficiency. The final concentration of GSI-1 was 5 μM, which was reported to be effective for suppressing r-secretase activity.[24] We cultured 60–70% confluent MCF7, empty vector, and ΔNp63α cells in the presence or absence of GSI-1 for 48 h before being assayed for colony formation in soft agar. Comparisons between the DMSO vehicle control and GSI-1 treatment showed GSI-1 significantly reduced colony forming efficiency of ΔNp63α cells ($22.6 \pm 1.2\%$ *vs* $8.7 \pm 0.5\%$; $P < 0.001$, Fig. 4E). MCF7 and empty vector cells were not significantly affected (Fig. 4E). These results show that ΔNp63α enriches the breast cancer stem cell subpopulation, at least in part, by activating Notch pathway.

ΔNp63 binds directly to Notch1. As ΔNp63α overexpression increased Notch1 expression most, we hypothesized that ΔNp63α might activate Notch1 promoter. Previous experiments have defined the optimal p63 DNA-binding consensus motif as (T/A)A(T)ACA(T)TGT(T/A)T, consisting of a CA(T)TG core and AT-rich 5′ and 3′ flanking sequences.[25] We obtained the genomic sequence of the human Notch1 gene from the GenBank

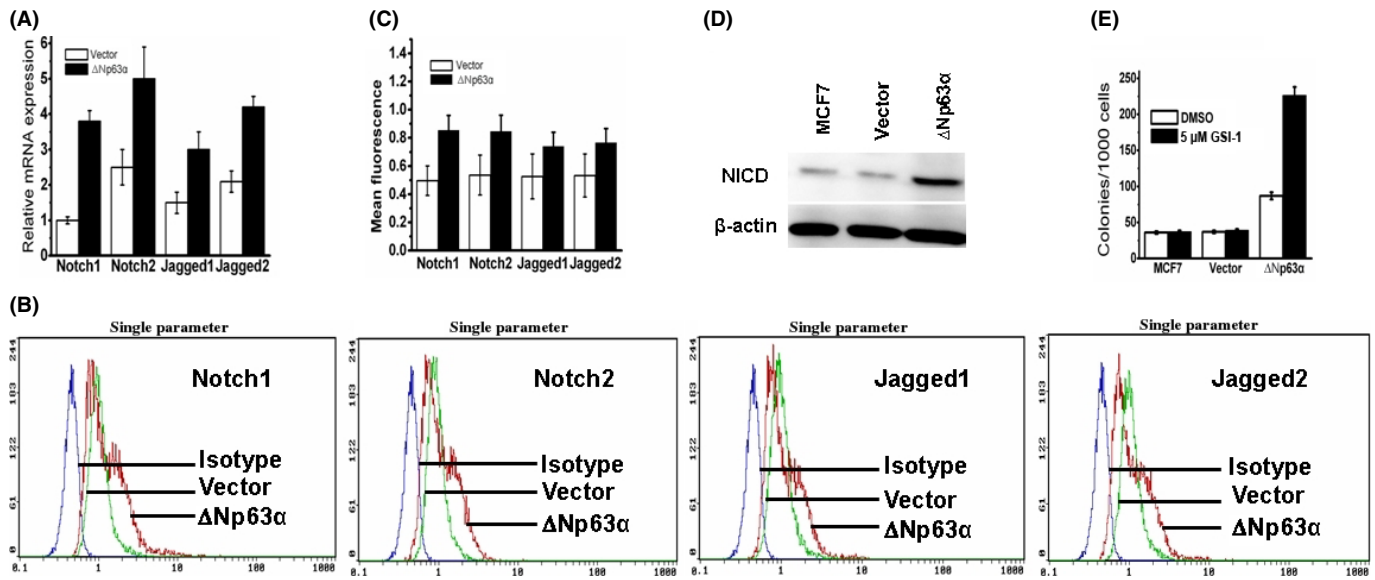

Fig. 4. ΔNp63α overexpression activates the Notch pathway in MCF7 breast carcinoma cells. (A) mRNA levels of Notch1, Notch2, Jagged1, and Jagged 2 were detected by quantitative RT-PCR with β-actin as the internal control. Fold expression values were normalized to vector cells. Data represent the mean ± SD of three independent experiments. (B) FACS histograms of representative experiments showing Notch1, Notch2, Jagged1, and Jagged 2 expressions of ΔNp63α cells (red line) and vector cells (green line) relative to an irrelevant isotype control (blue line). (C) Relative fluorescence of Notch1, Notch2, Jagged1, and Jagged2 was calculated by normalizing all data to the fluorescence of corresponding isotype control cells. Notch1 and Notch2 were significantly increased in ΔNp63α cells compared to vector cells ($P < 0.05$). Expression levels of Jagged1 and Jagged2 were similar ($P > 0.05$). Data represent the mean ± SD of three independent experiments. (D) Western blot analysis of notch-1 intracellular domain (NICD) showed a concordant accumulation in ΔNp63α cells. β-actin antibody was used to verify differences in protein loading in each lane. (E) The colony formation efficiency was significantly decreased in ΔNp63α cells treated with GSI-1.

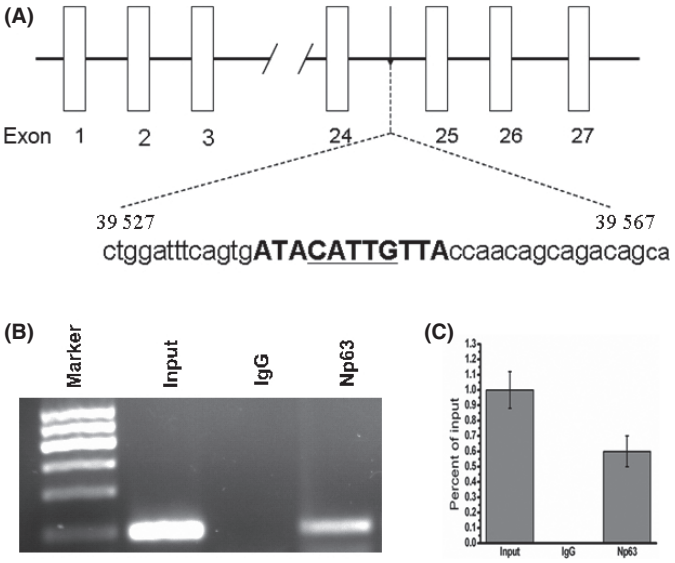

Fig. 5. Chromatin immunoprecipitation assay for Np63 and Notch1. Regulation of Notch1 expression by p63. (A) The potential responsive site of the *Notch1* gene, RE-Notch1, is located at the 24th intron of the *Notch1* gene and consists of a CA(T)TG core of the p63-binding sequence and AT-rich 5′ and 3′ flanking sequences. (B) P63 interacts with the RE-Notch1 sequence. Chromatin immunoprecipitation assay of a genomic fragment (nucleotide position 39509–39627, where 1 represents the translation initiation site). IgG and GAPDH served as negative and positive controls, respectively. (C) Quantitative ChIP of anti-p63. Error bars represent SD for three independent experiments.

database (Accession No. NC_000009.11) and searched for a similar sequence. We identified a candidate sequence at the position 39540, where 1 represents the translation initiation site, then carried out a ChIP assay. Our results showed that ΔNp63 could directly bind to Notch1 (Fig. 5). We assayed p63 and Notch1 mRNA levels in the $CD24^{-}CD44^{+}$ subpopulation from three primary breast cancer specimens. The $CD24^{-}CD44^{+}$ subpopulation had a higher p63 and Notch1 expression level than other populations (Fig. 6A). Western blot analyses showed that p63 correlated with Notch1 expression in several breast cancer cell lines (Fig. 6B). We then compared the Notch1 expression levels between $p63^{+}$ and $p63^{-}$ primary tumors. We found that p63 overexpressing specimens usually had a higher Notch1 expression level than $p63^{-}$ specimens (Fig. 6C, Table 4).

Discussion

As published in previous reports,[15,26] we found that p63 was highly expressed in a subtype of breast cancer patients, characterized by the absence of ER, PR, and HER2 expression. These ''triple negative'' breast cancers are mainly comprised of basal-type breast cancer, which usually has a poor prognosis and is considered to have breast stem cells. As breast cancer might result from transformation of normal breast stem cells, and ΔNp63α plays a critical role in normal breast stem cells,[12] we next investigated the role of ΔNp63α in breast cancer cell fate. As Np63 is expressed in MCF7 and its expression level is low compared with the triple negative breast cancer line, we overexpressed ΔNp63α in the MCF7 breast cell line. When ΔNp63α was overexpressed in MCF7 cells, the percentage of

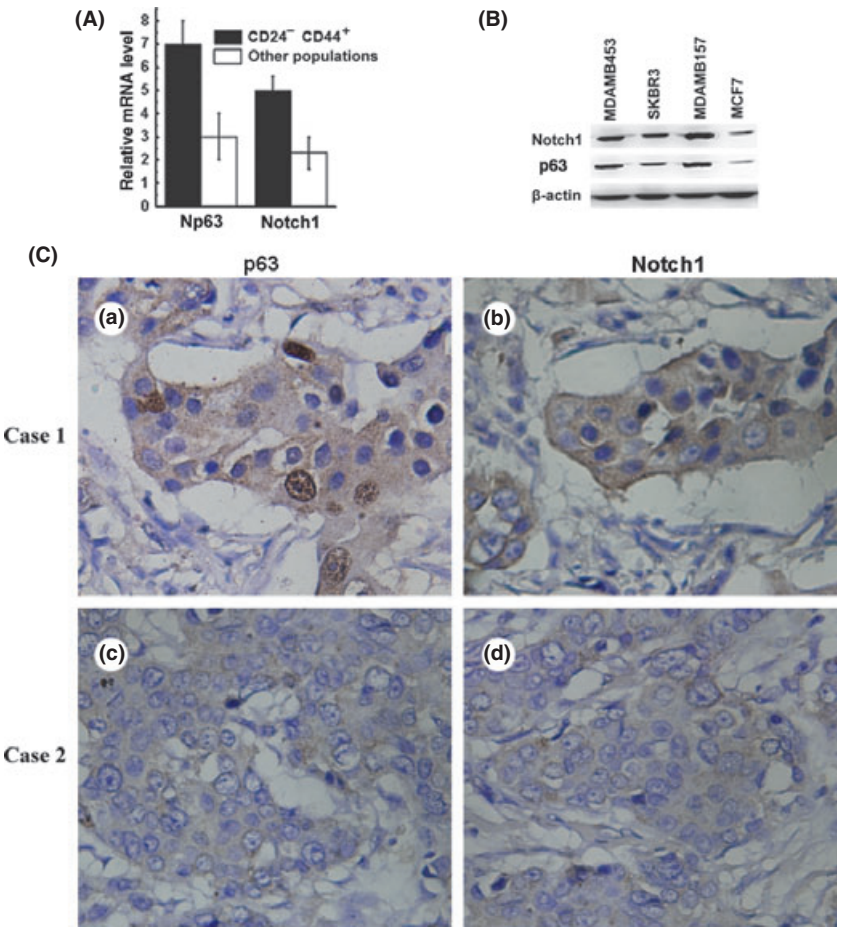

Fig. 6. P63 correlates with Notch1 expression in breast cancer cell line and in primary breast tumors. (A) Flow cytometry was used to isolate $CD24^{-}CD44^{+}$ cells and other populations from primary breast cancer specimens and p63 and Notch1 mRNA level were detected by quantitative RT-PCR with β-actin as the internal control. (B) Western blot of p63 and Notch1 in four breast cancer cell lines. β-actin antibody was used to verify differences in protein loading in each lane. (C) $P63^{+}$ tumors have higher Notch1 expression. Representative of specimens positive for both p63 (a) and Notch1 (b). Representative specimens negative for both p63 (c) and Notch1 (d). Magnification, ×400.

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Table 4. Relationship between p63 and Notch1 expression in MCF7 breast carcinoma cells

Feature	$p63^+$	$p63^-$	*P*-value
Total	8	42	NA
Notch1			
Negative	0	3	$P < 0.001$
Low positive	1	37	NA
High positive	7	2	NA

P-value was calculated using the χ^2-test and Fisher's exact test. *P*-value was considered to be statistically significant at $P < 0.05$ (two-sided). NA, not applicable.

$CD24^-CD44^+$ subpopulation was increased, indicating that ΔNp63α is critical for breast cancer stem-like cells.

A previous study has shown that MCF7 cells can form mammospheres in serum-free low attachment plates. These mammospheres are enriched for breast cancer stem cells and progenitor cells.[27] ΔNp63α overexpression could increase the mammosphere forming efficiency of MCF7 cells. Double immunofluorescence staining showed that these mammospheres were mainly comprised of $CD24^-CD44^+$ cells. In addition, ΔNp63α overexpressing MCF7 cells could form more colonies in soft agar. They were more drug resistant than their parental cells. Most importantly, ΔNp63α cells were more tumorigenic after being transplanted into NOD/SCID mice. Thus, overexpression of ΔNp63α can induce a stem cell phenotype in MCF7 cells.

The role of ΔNp63α in cancer development is still not very clear. Adorno *et al.*[14] showed that ΔNp63 served as a tumor suppressor to oppose transforming growth factor (TGF)-β-induced metastasis in breast cancer. In squamous epithelial malignancies, ΔNp63 served as a survival factor to antagonize TP73-induced apoptosis.[28] It has also been indicated that ΔNp63 was an essential survival factor for breast cancer.[13] Our findings suggest that ΔNp63α plays an important role in breast cancer stem-like cells. Overexpression of ΔNp63α could increase the subpopulation of breast cancer stem-like cells. This is consistent with a recent finding that knockdown of p63 makes cancer stem cells lose their self-renewal ability.[29]

The Notch pathway is evolutionarily conserved with an important role in cell fate determination and differentiation. There are four mammalian Notch genes (Notch1–4), which encode a single transmembrane receptor. The Notch pathway is activated through the interaction of Notch receptors with Δ-like and Jagged ligands on neighboring cells.[30] This leads to proteolytic cleavages, which release the Notch intracellular domain. The Notch pathway has been implicated in self-renewal of stem cells, including stem and/or progenitor cells isolated from the mammary gland.[24,31] In addition, overexpression of active forms of the Notch1 and Notch4 receptors transform both normal human and murine mammary epithelial cells.[32–34] Similar to the report that p63 could activate the Notch pathway in keratinocytes,[22] we found that ΔNp63α overexpression in MCF7 cells increased mRNA and protein levels of Notch1 and Notch2. Notch1 intracellular domain was accumulated. Treatment of ΔNp63α overexpressing cells with GSI-1, a Notch pathway inhibitor, could markedly block the effects of ΔNp63α, as indicated by decreased colony forming efficiency. These results suggest that ΔNp63α enriches the breast cancer stem-like subpopulation mainly through activating the Notch pathway. This may explain why basal-like breast cancer cells express higher levels of Notch ligands than ductal breast cancer cells.[35]

In keratinocytes, Sasaki *et al.*[22] identified a specific binding site for the p63 protein in the second intron of the *Jagged1* gene and showed that p63 could activate Notch signaling to the neighboring cells. Birkaya *et al.*[23] found that Np63 could directly activate Notch2. In our experiments, we showed that ΔNp63α overexpression could cause a marked increase of Notch1 expression in breast cancer cells by directly transactivating Notch1.

We found that MCF7 cells overexpressing ΔNp63α were drug resistant. We did not carry out further investigation into the mechanism underlying this phenomenon. But ΔNp63 isotypes were shown to be able to transcriptionally regulate heat shock protein 70 expression, multidrug resistant gene 1, which might account for their chemoresistance.[36,37]

Breast cancer is considered a stem cell disease, which arises from mutated normal stem cells. With self-renewal ability, adult stem cells usually have a long lifespan, which means they are exposed to more damaging agents and have more chance to mutate. P63 plays an important role in breast development, which was evidenced by a transgenic mouse model and limb mammary syndrome.[38] Our results suggest that basal-type breast cancer might result from transformation of breast stem cells, and ΔNp63 could be a potential target for breast cancer.

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