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14. ABSTRACT: Ectopic delta-N-p63 could block retinoic acid induced differentiation in embryonic carcinoma cells NT2/D1, and preserve transcript level of nestin post RA treatment. Similarly, RA treatment could inhibit the proliferation of breast cancer cell lines, and down-regulate the mRNA level of some self-renew relative genes including oct3/4, nanog and dab2 in these cells. Immunocytofluorescence staining detected existence of delta-N-p63 in both estrogen receptor negative cells such as SUM102, SUM149 and MDA-MB-231 cells, but also MCF-7 cells with luminal epithelial phenotype. And delta-N-p63 positive cells are not well-differentiated and lost expression of cell cycle marker ki-67, cyclin D1. Infection of breast cancer cells with delta-N-p63 adeovirus could decrease cell growth rate, cause G1/G0 cell phase arrest. In MCF-7 cell, ectopic delta-N-p63 could induce cells to lost expression of ki-67 and cyclin D1. Semi-quantitative PCR assay showed that over expression of delta-N-p63 had diverse effect on transcript level of some self-renew correlating gene events such as TA-P63, oct3/4, nanog, sonic hedgehog, hTERT, ect.					
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Introduction

Recent studies support a tumor stem cell theory, which holds that tumor causative lesions are happened in multi-potent progenitor with retained proliferative capacity and a prolonged lifespan. Throughout female reproductive life the epithelial portion of the mammary gland undergoes multiple periodic regenerative cycles characterized by cellular proliferation and terminal differentiation (1). Continuous regenerative cycling of the epithelial portion of the mammary gland depends upon a subset of Self-Renewing Basal Progenitors (SRBPs) that retain their proliferative capacity and resist terminal differentiation (2). These features confer a prolonged replicative lifespan indicating that such progenitor cells may be capable of accumulating mutations and harboring them from the protective effects of apoptosis. In addition, such mammary progenitor cells may be the sites of breast cancer initiation, which is supported by studies in which mammary tumor stem cells were prospectively identified and shown to be uniquely tumorigenic and able to self-renew (3). These studies suggested that cancer initiation is a condition of unregulated or poorly regulated self-renewal and have focused attention upon potential of genetic pathways that regulate self-renewal. The aim of our proposal is to localize genes associated SRBPs' self-renewal or differentiation, which have prognostic value and may enhance the ability of clinicians to design therapeutic strategies that are specific to individual patients.

There has been abundant evidence that the p53 family member TP63 plays a critical role in making decision to preserve or forfeit mammary progenitor cells' self-renewing capacity (4-7). The gene encoding TP63 utilizes proximal and distal promoters to produce Trans-Activating (TA-p63) and N-terminally deleted (Δ N-p63) isoforms (8). In adult mammary gland, abundant data reveal that expression status of TP63 reflects preservation or forfeiture self-renewing capacity (9). Mutations in TP63 have been shown to underlie a broad spectrum of syndromes, such as Limb-Mammary Syndrome that have in common defects in the establishment or cellular stasis of a variety of epithelial and apocrine structures (6, 10). These defects are believed to underlie a genetic program of non-regerative differentiation that ultimately leads to the depletion of progenitor pools.

Targeted ablation of TP63 in the mouse resulted in profound failure of both embryonic and adult epithelial and apocrine structures (5, 7). Additionally, studies using pan-p63 antibody (4A4) indicate that TP-p63 predominates in the basal epithelia of mammary gland, suggesting that TP-63 may play an important role in the preservation of mammary progenitors (11). This is further supported by studies indicating that TP-63 is a marker of progenitor cell population in corneal keratinocytes and that repression of TP-63 expression is correlated with the transition to a transient amplifying cell population and terminal population (12). One isoform specific antibodies which could distinguish delta-N-p63 specifically has been generated in our lab, facilitating further study the role of Δ N-p63 in progenitor cells biological behavior dramatically (13).

It has been reported that delta-N-p63 co-localizes with some other regenerative cellular marker, such as nestin, in breast basal epithelial cells. Furthermore, immunohistochemistry analysis of basal epithelial breast tumors indicates robust expression of nestin and punctuate expression of delta-N-p63 in such breast tumor subtype, implicating the selective role of nestin and delta-N-p63 in basal epithelial breast cancer subtypes including BRCA-1 mutation carrying tumors. On the other hand, in the embryonal carcinoma cell line NT2/D1, abundant with nestin, ectopic delta-N-p63 could disrupt retionic acid-induced differentiation, thereby preserving expression of nestin. All of these suggest delta-N-p63 might play a key role in the multi-potential progenitor self-renewal process collaborating with other important regeneration related genes including nestin. And such genes have highly potential diagnostic and prognostic evaluation values in breast cancer due to the definitive role of delta-N-p63 in normal mammary gland regenerative compartment and basal epithelia breast tumor subtype. Our study would focus on the biological function of delta-N-p63 in breast tumor and further investigate its influence on other self-renewing biomarkers, which could be the promising candidates for clinical diagnosis and outcome evaluation.

Body:**Materials and Methods:**

NT2/D1 tissue culture, Northern blot analysis of nestin and differentiation marker A2B5 staining: See publication titled with “ Nestin Is Expressed in the Basal/Myoepithelial Layer of the Mammary Gland and Is a Selective Marker of Basal Epithelial Breast Tumors” (Li H. et al.), Cancer Research, January 15, 2007, p501-510.

Breast cancer cell line tissue culture: The breast cancer cell line including MCF-7, SUM102, SUM149, MDA-MB-231 and MDA-MB-468 were cultured as described previously. The culture media of MCF-7, MDA-MB-231 and MDA-MB-468 was DMEM media complemented with 10% fetal bovine serum; the medium of SUM 149 cells was Ham's F12 media supplemented with 10% fetal bovine serum, 10 mM HEPES, 5 ug/ml insulin, 1 ug/ml hydrocortisone; the culture medium of SUM102 was Ham's F12 supplemented with 10 mM HEPES, 5 ug/ml insulin, 1 ug/ml hydrocortisone, 10 ng/ml EGF. All cell lines were cultured at 37°C, 5% CO₂ incubator. For differentiation experiments, all cell lines were treated with 1umol/L all-trans-retinoic-acid and 0.01% DMSO as vehicle control. For delta-N-p63 overexpression experiments, all breast cancer cell lines were infected with delta-N-p63 alpha and GFP empty vector as vehicle control with ratio at 1ul/ml.

Immunocytofluorescence staining: The breast cancer cell lines were plated onto cover slides in 6-well plates and cultured for overnight. Then culture medium was removed and the slides were washed with PBS (pH7.0) twice. The cells were fixed with 3.7% paraformaldehyde at room temperature for 15 min. After washing with PBS, cells were blocked with 5% serum/PBS at 37°C for 20 min. The blocking serum was removed, and the cells were incubated with primary antibodies in 1% serum/PBS (pan-p63 (4A4), delta-N-p63, Ki-67, cyclin D1 and estrogen receptor) at 37°C for 45 min . For two-color immunofluorescence staining, two antibodies from different host species could be incubated together. Then the slides were washed with PBS for 5 min, 3 times. The cells were further incubated with secondary antibodies in 1% serum/PBS at 37°C for 30 min. Then the slides were washed with PBS for 5 min, 3 times and mounted with 50%

glycerol/DAPI. All the applied primary and secondary antibodies are listed as follows: pan-p63 (4A4), mouse-anti-human, 1:100; delta-N-p63, rabbit-anti-human, 1:200; ki-67: rabbit-anti-human, 1:100; cyclin D1, rabbit-anti-human, 1:50; estrogen receptor, rabbit-anti-human, 1:50; Alex Fluorescence-594 goat anti rabbit, Alex Fluorescence-594 goat anti mouse, Alex Fluorescence-488 goat anti rabbit, Alex Fluorescence-488 goat anti mouse are all diluted at 1:200.

Flowcytometry PI-staining: The breast cancer cell lines were treated with 1 $\mu\text{mol/L}$ RA and 0.01% DMSO, infected with delta-N-p63 and GFP empty vector respectively. With such treatment for 72 hours, the cells were collected by trypsinization. The harvested cells were fixed with 70% ethanol/PBS on ice for 30 min, and incubated with 2.5 mg/ml RNase A at 37°C for 45 min. Then added propidium iodide in PBS (final concentration at 0.05 mg/ml) and incubated at 37°C for another 30 min. Then the cells were measured with flowcytometry to analyze cell cycle.

Cell counting: All breast cancer cell lines were plated into 10 cm culture dishes at $10^4/\text{ml}$ cellular density. The second day, the cells were treated with 1 $\mu\text{mol/L}$ RA and 0.01% DMSO, infected with delta-N-p63 and GFP empty vector respectively. All the cell samples were harvested after 72 hours treatment and were counted directly.

Quantitative PCR and semi-quantitative PCR: All applied breast cancer cell lines were treated with 1 $\mu\text{mol/L}$ RA and 0.01% DMSO, infected with delta-N-p63 and GFP empty vector respectively. Total RNA samples were collected with trizol reagent, and 0.5 μg RNA was applied for making cDNA with reverse transcriptase kit (Invitrogen). All applied genes primers are listed as follows: delta-N-p63: forward 5'-ATG TTG TAC CTG GAA AAC-3'; reverse 5'-ATG GGG CAT GTC TTT GC-3'; TA-p63: forward 5'-GAT CGA ATT CAT GTC CCA GAG CAC ACA G-3'; reverse 5' GAT CAA GCT TCC ACA TGG GGT CAC TCA-3'; sonic hedgehog: forward 5'-GAA AGC AGA GAA CTC GGT GG-3'; reverse 5'-GGA AAG TGA GGA AGT CGC TG-3'; oct-3/4: forward 5'-CGA CCA TCT GCC GCT TTG AG-3'; reverse 5'-CCC CCT GTC CCC CAT TCC TA-3'; nanog: forward 5'-CAA AGG CAA ACA ACC CAC TT-3'; reverse 5'-CTG GAT GTT CTG GGT CTG GT-3'; dab2: forward 5'-ACA AGT GCA ACC AAT GGT CA-3'; reverse 5'-TCC TCC ACA CAC GTA ACC AA-3'; musashi: forward 5' CGA ACG AAG AAG ATC TTT CTG-3'; reverse 5'-TCC

GTA GGG CAT GAC TCG AGA-3'; sox-2: forward 5'-AGA ACC CCA AGA TGC ACA AC-3'; reverse 5'-ATG TAG GTC TGC GAG CTG GT-3'; Rex-1: forward 5'-GGA GGA ATA CCT GGC ATT GA-3'; reverse 5'-TTA GGA TGT GGG CTT TCA GG-3'; TERT(telomerase reverse transcriptase element): forward 5'-AAC GTT CCG CAG AGA AAA GA-3'; reverse 5'-AAG CGT AGG AAG ACG TCG AA-3'; cytokeratin 14: forward 5'-AGA TTC TCA CAG CCA CAG TGG ACA-3'; reverse 5'-TGA AGA ACC ATT CCT CGG CAT CCT-3'; cytokeratin 17: forward 5'-CAG TTC ACC TCC TCC AGC TC-3'; reverse 5'-AAC TTG GTG CGG AAG TGA TC-3'; cytokeratin 19: forward 5'-TTT GAG ACG GAA CAG GCT CT-3'; reverse 5'-TCT TCC AAG GCA GCT TTC AT-3'; GAPDH: forward 5'-AAG GTC GGA GTC AAC GGA TTT GGT-3'; reverse 5'-AGT GAT GGC ATG GAC TGT GGT CAT-3. For quantitative PCR, the reaction system was: 12.5 ul Sybergreen Supermix reagent (Bio-rad), 0.5 ul sense primer (10umol/L), 0.5 ul antisense primer (10umol/L), 0.5 ul cDNA, 11.0 ul water. For semi-quantitative PCR, the reaction system was: 2.5 ul PCR buffer (10 ×), 1.0 ul MgCl₂ (2.5 mmol/L), 0.5 ul dNTPs (10mmol/L), 0.5 ul sense primer (10umol/L), 0.5 ul antisense primer (10umol/L), 0.2 ul Tag polymerase (Invitrogen), 0.5 ul cDNA, 19.3 ul water. And the PCR program is 94°C for 10 min, then 94°C 30s, 56°C 45s, 72°C 1min for 40 cycles, 72°C for 10 min, then store samples at 4°C.

Results:

Delta-N-p63 blocks cellular differentiation and preserves nestin expression in embryonal carcinoma NT2/D1 cells

See publication titled with " Nestin Is Expressed in the Basal/Myoepithelial Layer of the Mammary Gland and Is a Selective Marker of Basal Epithelial Breast Tumors" (Li H. ect.), Cancer Research, January 15, 2007, p501-510.

Delta-N-p63 is expressed in some breast cancer cell lines which displaying basal or luminal epithelial phenotype.

Most breast cancer cell lines are characterized with basal epithelial phenotype, and delta-N-p63 has been proven to be a definitive biomarker for normal breast epithelial cells

in mammary gland regenerative compartment and basal/myoepithelial breast cancer subtypes. Such observations suggest that delta-N-p63 may exist in some breast cancer cell lines, especially in the cells with basal epithelial phenotype. Pan-p63 and delta-N isoform specific antibodies were applied to detect the expression of delta-N-p63 in breast cancer cell lines including SUM102, SUM149, MCF-7, MDA-MB-231 and MDA-MB-468. Immunocytofluorescence staining showed that there were positive staining signals of pan-p63 and delta-N-p63 antibodies in SUM102, SUM149, MCF-7, MDA-MB-231, not in MDA-MB-468. For further clarify the phenotype of these five breast cancer cell lines, expressions of cytokeratin markers including 5, 14, 17, 18, 19 were observed by immunocytofluorescence. The staining pattern indicated SUM102 displayed typical basal epithelial phenotype such as cytokeratin 5, 14, 18 positive and cytokeratin 19 negative; and MCF-7 demonstrated regular luminal epithelial phenotype such as cytokeratin 19 positive, but cytokeratin 5, 14 and 18 negative. Interestingly, there was p63 staining positive cells in both SUM102 and MCF-7 cells although they showed different epithelial phenotype. To further confirm the specificity of delta-N-p63 expression in these cell lines, two-color immunofluorescence was employed to detect the degree of overlapping between pan-p63 (4A4) and delta-N-p63 isoform specific antibodies' staining. Clearly, there were smaller population cells could be double stained with both 4A4 and delta-N isoform specific antibodies in MCF-7 and SUM102 cell lines, which implicating the existence of delta-N-p63 in these cell lines, rather than other p63 isoforms. Moreover, the distribution pattern of delta-N-p63 positive cells is not universal, but punctuate in these breast cancer cell lines, suggesting heterogeneity of most breast cancer cells. The total RNA was collected from all these cell lines and further quantitative PCR analysis was applied for evaluating the transcript level of delta-N-p63. Consistent with immunofluorescence staining, delta-N-p63 mRNA could be detected in SUM102, SUM149, MCF-7, MDA-MB-231, not in MDA-MB-468 cells.

The delta-N-p63 positive cells in MCF-7 cell line are not well differentiated and lose expression of cellular marker ki-67 and cyclin D1.

We have successfully detected the expression of endogenous delta-N-p63 in estrogen

receptor positive breast cancer cell line MCF-7. The phenotype profiling has proven its luminal epithelial phenotype, which demonstrated with robust expression of luminal epithelial marker, cytokeratin 19 and undetectable protein level of basal/myoepithelial marker, cytokeratin 5, 14. Moreover, there is only small set of population cells expressing endogenous delta-N-p63, which implicating that such delta-N-p63 positive cells having different biological attributes from other delta-N-p63 negative tumor cells. In normal breast ducts, delta-N-p63 is not co-localized with estrogen receptor, which just exists in well differentiated, cytokeratin 19 positive luminal epithelial cells. Two-color immunocytofluorescence staining of delta-N-p63 and estrogen receptor was utilized to examine biological phenotype of such delta-N-p63 positive tumor cells. Our staining indicated that there is no any delta-N-p63 and estrogen receptor co-existing cell in MCF-7 cell line, which obviously suggesting the delta-N-p63 positive cells are not ended differentiated luminal epithelial cells. Ki-67 and cyclin D1 are two well-recognized cell cycle biomarker, and loss of their expression usually indicates that cell leaves cellular cycle. Current studies have revealed that most of self-renewing multi-potential progenitor cells are retained at quiescent stage, resistant to cellular differentiation but neither re-enter into cell cycle nor be programmed to apoptosis. It has been discovered that delta-N-p63 positive cells are not differentiated and apoptotic, which raise the possibility that such small portion cells could be constrained in quiescent cell phase for maintaining self-renewal capacity. To test the cell cycle property of these delta-N-p63 positive cells, co-staining of delta-N-p63 and ki-67, cyclin D1 was employed to detect the degree of overlapping between delta-N-p63 and these cell cycle markers in MCF-7 cells. Interestingly, we failed to detect any co-existence between delta-N-p63 and ki-67 or cyclin D1 in MCF-7 cells, revealing that delta-N-p63 positive cells had lost expressions of important cell cycle biomarkers, and left proliferative cell cycle. Taken together the separate distribution of delta-N-p63 and estrogen receptor in MCF-7 cells, it is apparently assumed that delta-N-p63 positive cells are quiescent cells with self-renewing capacity.

Both retinoic acid treatment and ectopic delta-N-p63 could decrease the growth rate of all breast cancer cells.

The treatment of retinoic acid could inhibit the proliferation of immortalized basal epithelial cells (IMECs), and cause IMEC cells to start differentiation. Removal of retinoic acid could not re-induce IMEC cells to enter into cell cycle, indicating that such differentiation –inducing effect was not reversible. Similarly, such biological influence of retinoic acid was also observed in embryonic carcinoma NT2/D1 cells recently. Multi-potential progenitor cells tend to have prolonged life and self-renewal capacity, which characterized with cellular ability to resistant to differentiation and re-enter into proliferative cellular phase. There have been plentiful evidence that delta-N-p63 plays a key role in such proliferation or differentiation decision-making process. We have detected the existences of delta-N-p63 protein and transcript in most breast cancer cell lines, including estrogen receptor negative ones and even MCF-7 cell line displaying luminal epithelial phenotype and having strong cytokeratin 19 and estrogen receptor staining. Such findings coupled to signature distribution of delta-N-p63 in normal breast basal epithelial cells and basal/myoepithelial breast cancer subtype, suggest that delta-N-p63 might be also essential to self-renew in breast tumor. To further study the biological function of delta-N-p63 in breast cancer cells, we over expressed ectopic delta-N-p63-alpha by infection breast cancer cell lines with GFP recombinant delta-N-p63 alpha specific adenovirus and GFP protein vector one as control. Five diverse breast cancer cell lines were included such as SUM102, SUM149, MCF-7, MDA-MB-231, MDA-MB-468. Total RNAs were harvested for quantitative PCR of delta-N-p63 to analyze infection efficiency after infection 72 hours. Cell counting and PI-staining flowcytometry were applied to determine the influence of such over expression of delta-N-p63 on cellular proliferation and cell cycle. Our cell counting data evidently showed that ectopic delta-N-p63 could decrease the proliferation rate in all infected breast cancer cell lines, and cell cycle analysis demonstrated that over expressed delta-N-p63 could induce more cells to enter into G₁/G₀ phase. Similarly, these breast cancer cell lines were treated with 1umol/L all-trans-retinoic acid and 0.01% DMSO for 72 hours. Then, all cell samples were analyzed with cell counting and PI-staining flowcytometry respectively. Compared with DMSO control group, RA treatment could inhibit the growth of breast cancer cell lines significantly, and increase the percentage of G₁/G₀ phase cells.

Overexpression of delta-N-p63 could induce the cells lose expression of cell cycle biomarker ki-67, cyclin D1 in MCF-7 cell line

In MCF-7 cell line, the delta-N-p63 positive cells are estrogen receptor negative and lose the expression of cell cycle biomarkers such as ki-67 and cyclin D1, implicating its possible quiescent cell phase. Further adenovirus infection analysis revealed that ectopic delta-N-p63 could slow down the proliferation rate of breast cancer cell lines including MCF-7 cells, which is possible due to ability of delta-N-p63 to induce cells to leave proliferative cell cycle. MCF-7 cells were infected with delta-N-p63-alpha and GFP protein vector adenovirus for 72 hours, and two-color immunocytofluorescence staining was applied to detect the expression of delta-N-p63 and cell cycle markers ki-67, cyclin D1. The staining clearly showed that infection of adenovirus could dramatically increase protein level of delta-N-p63 in MCF-7 cells, and the overlapping between delta-N-p63 and ki-67, cyclin D1 was not detectable in infected MCF-7 cells. Further flowcytometry analysis revealed that the percentages of ki-67, cyclin D1 positive cells in delta-N-p63 adenovirus infected group was less than those of GFP protein vector infected group significantly. To further determine the influence of ectopic delta-N-p63 on expression of cell cycle biomarkers ki-67 and cyclin D1 in MCF-7 cells, GFP positive and negative cells in delta-N-p63 and empty vector adenovirus infected MCF-7 cells were sorted with flowcytometry respectively and analyzed with two-color immunofluorescence staining post cytopinning. In delta-N-p63 infected MCF-7 cells, most of green cells were delta-N-p63 stained positively, and ki-67, cyclin D1 negatively; while most of non-green cells were delta-N-p63 stained negatively, and ki-67, cyclin D1 positively. Such difference was not detected in GFP empty vector infected MCF-7 cells. Such discoveries coupled to ectopic delta-N-p63 could inhibit the growth of breast cancer cell line and en-longer their cell cycle, implicating that delta-N-p63 could induce proliferative cells to leave cell cycle and retain at quiescent stage for their self-renewal capacity.

The retinoic acid treatment has different influence on self-renewing biomarkers in NT2/D1 cells and some breast cells.

Our studies have shown that retinoic acid could down-regulate expression of delta-N-p63 in immortalized basal epithelia cells (IMEC), and loss of delta-N-p63 could lead up to forfeiture of cellular self-renewal capacity, and irreversible cellular differentiation. In embryonic carcinoma NT2/D1 cells, ectopic delta-N-p63-alpha could block retinoic acid induced differentiation and preserved the expression of nestin. All these findings suggest delta-N-p63 could prevent from progenitor cells from retionic acid induced differentiation to keep cellular self-renewal ability. NT2/D1 cells, IMCE cells and other breast cancer cell lines including SUM 102, SUM149, MDA-MB-231, MDA-MB-468 and MCF-7 cells were treated with 1umol/L retinoic acid and 0.01% DMSO as vehicle control. Total RNA was collected at 0h, 24h, 48 h, 72 and 96h after RA or DMSO treatment, then quantitative PCR was used to detect the transcript level of some self-renewal biomarkers post retinoic acid treatments. In NT2/D1 cells, RA treatment could decrease transcript level of oct-4, nanog and increase message level of dab2 and musashi; in IMEC cells, RA treatment could decrease transcript level of delta-N-p63, oct-4, nanog and dab2; in sum 149 cells, RA treatment could down-regulate transcript level of delta-N-p63, but increase message level of oct3/4, nanog and dab2; in MDA-MB-231 cells, RA treatment could increase transcript level of delta-N-p63 and nanog, but decrease mRNA level of nanog and dab2; in MCF-7 cells, RA treatment has not dramatic effect on transcript level of most self-renewal relative genes including delta-N-p63, oct3/4, nanog, dab2. The effect of retinoic acid treatment on transcript level of other phenotype biomarkers such as cytokeratin 14, 17 and 19 were also analyzed with quantitative PCR. Our study showed that RA treatment could decrease mRNA level of cytokeratin 14, 17 in both IMEC and SUM 149 cells, but has not dramatic effect on message level of cytokeratin 19.

Overexpression of delta-N-p63 has diverse effect on transcript level of some self-renewal relating genes

Our study has demonstrated that ectopic delta-N-p63 could inhibit the proliferation and alter cell cycle of most breast cancer cell lines. Further immnofluescence staining revealed that delta-N-p63 could induce tumor cells to leave proliferative cell cycle and lose expression of cell cycle biomarkers ki-67 and cyclin D1. In NT2/D1 cells, ectopic

delta-N-p63 also could block RA-induced differentiation by preserving transcript level of nestin. Taken together, it is assumed that delta-N-p63 have some influence on message level of some self-renew markers. We tested the effect of delta-N-p63 on some candidate genes with semi-quantitative PCR, and the examined genes were included TA-p63, sonic hedgehog, oct3/4, nanog, dab2, musashi, sox-2, Rex-1 and human TERT. Our preliminary PCR data displayed that over expression of delta-N-p63 could increase transcript level of TA-p63 in MCA-10A, SUM102, MDA-MB-231, MDA-MB-468 cells, but decrease TA-p63 mRNA level in SUM149 cell. The ectopic delta-N-p63 could increase sonic hedgehog message level in MCF-10A, SUM102, MCF-7 cells, while decrease its transcript level in MDA-MB-231, MDA-MB-468 cells, and has not any such dramatic effect in SUM149 cell. In terms of oct3/4, ectopic delta-N-p63 could increase its transcript level in SUM149 and MDA-MB-231 cells, down regulate its message level in MDA-MB-468 cells and has no significant effect in other breast cell lines; With respect to transcript level of nanog, over expressed delta-N-p63 could enhance its transcript level in MCF-10A, SUM102, MCF-7 cells, and decrease its message level in MDA-MB-231 and MDA-MB-468 cells, has not obvious effect in SUM 149 cells. Rex-1 mRNA was only detected in MDA-MB-468 cells, and delta-N-p63 could decrease its transcript level evidently. And ectopic delta-N-p63 could enhance transcript level of hTERT in MCF-10A, SUM149 and MDA-MB-231 cells.

Future directions:

The aim of our research is to identify genes events with clinical diagnostic and prognostic evaluation value. In our publication in Cancer Research, we showed that p63 is more important than p53 in such localization process in breast cancer due to the definitive role of p63, especially delta-N-p63 in normal breast basal epithelial cell and basal/myoepithelial breast cancer subtype. By screening of genes co-localizing with delta-N-p63 in normal breast regenerative compartment, novel basal epithelia like breast cancer diagnostic biomarker was discovered. And embryonic carcinoma cell line NT2/D1 was further employed to explore the biological function correlation between delta-N-p63 and such gene candidates. We are interested to follow up same study system to localize more

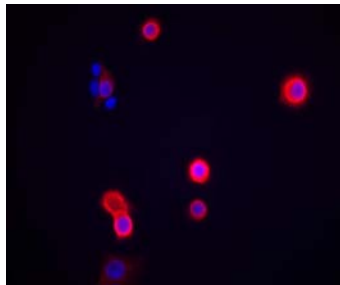
promising gene events collaborating with delta-N-p63 in multi-potential progenitor cellular self-renew process, which is essential to maintain the normal breast regenerative compartment and also important for breast cancer initiation. Given that there is no endogenous delta-N-p63 in NT2/D1 cells, our research extended to localize more breast cancer cell lines with endogenous delta-N-p63, which facilitate biological function study of delta-N-p63 in breast cancer. Our preliminary data has clearly demonstrated delta-N-p63 existed not only in SUM102, SUM149, MDA-MB-231 cells without estrogen receptor expression, but also MCF-7 cells with strong expression of cytokeratin 19 and estrogen receptor and definite luminal epithelial phenotype. Further analysis of biological character of these delta-N-p63 positive cells in MCF-7 cell line showed that there is no co-existence between delta-N-p63 and estrogen receptor, ki-67 and cyclin D1. All these observations implicated that delta-N-p63 positive cells are a unique subset of whole cell population. TP63 has been proven to play an essential role in stem cell or multi-potential progenitor cell's self-renewal process in diverse tissues. With respect to normal breast and breast cancer, it is assumed to delta-N-p63 is key to make decision about proliferation and differentiation in stem cell and progenitors. We are interested in perform thorough analysis of delta-N-p63 biological function in breast basal epithelial cells and breast tumor cells, then further identified gene candidates involving delta-N-p63 function mechanism and evaluate their correlation with clinical breast cancer diagnosis as well as prognosis evaluation. We have performed gain-function assay of delta-N-p63 in some breast cancer cell lines such as MCF-7, SUM102, SUM149, MDA-MB-231 and MDA-MB-468. Our data revealed that over expression of ectopic delta-N-p63 could inhibit proliferation rate of breast cancer cell line and induce more cells into more G₁/G₀ phase, lose expression of cell cycle marker ki-67 and cyclin D1. All these data suggested that delta-N-p63 could force breast cancer cells enter into quiescent stage. Currently, Choest and Pyronin Y staining is the most common method to distinguish cells in G₀ phase, and we need to further confirm delta-N-p63 over expressed cells are really in G₀ phase with such technology. Meanwhile, retinoic acid treatment also could contribute to cell G₁/G₀ arrest and cause further cellular differentiation. Therefore, it is necessary to localize more reliable biomarker to discern the difference between quiescence and differentiation in G₀

phase. In addition, we also plan to conduct experiments to elucidate the mechanism of delta-N-p63's attribute to force cells into quiescent phase to retain self-renewal ability. In terms of genes events sensitive to ectopic delta-N-p63 over expression, we have tested some self-renewal relative candidate genes including included TA-p63, sonic hedgehog, oct3/4, nanog, dab2, musashi, sox-2, Rex-1 and human TERT. And our preliminary data demonstrated that delta-N-p63 could regulate transcript level of these genes in some breast cancer cell lines. Further quantitative PCR and Western blot assay are needed to perform to clarify the biological regulation effect of delta-N-p63 on these candidate genes. Eventually, all promising genes would be tested on normal human breast tissue and diverse breast cancer subtypes samples.

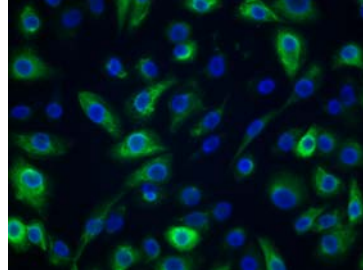
Supporting data

1. The phenotype of breast cancer cell lines:

Cytokeratin 14 expression in breast cells:



SUM102

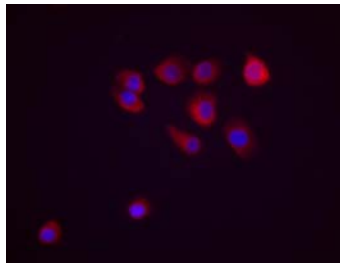


IMEC

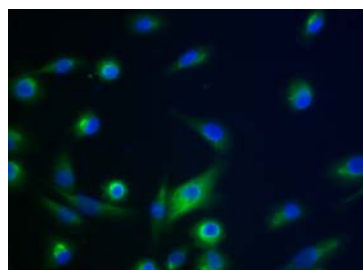


SUM149

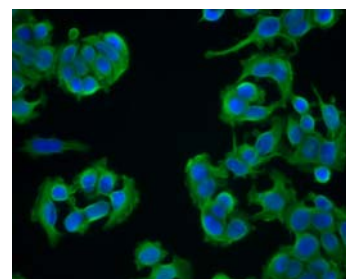
Cytokeratin 18 expression in breast cells:



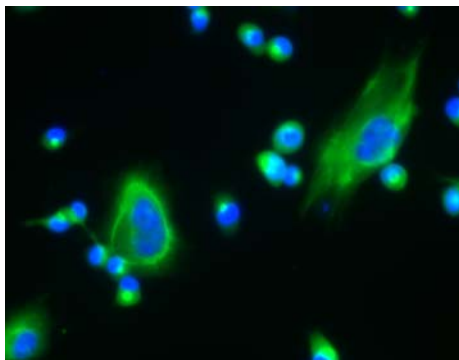
SUM102



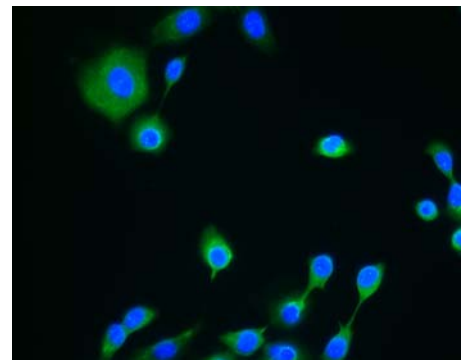
IMEC



MCF-7

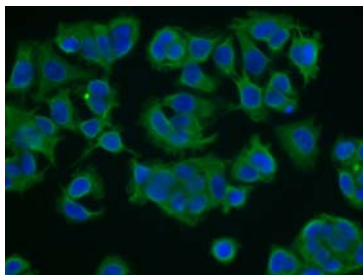


MDA-MB-231

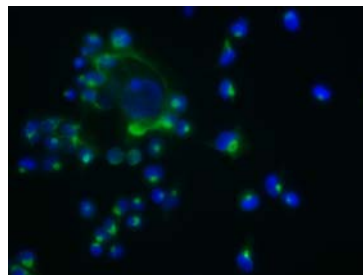


SUM149

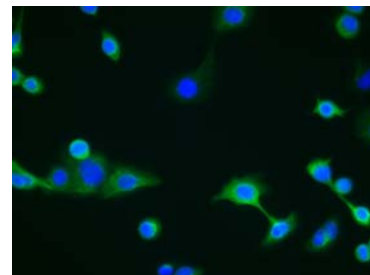
Cytokeratin 19 expression in breast cancer cells:



MCF-7



MDA-MB-231



SUM149

Figure 1: Immunocytofluorescence was applied to detect cytokeratin 14,

18 and 19 expression in immortalized breast basal epithelial cells (IMEC) and breast cancer lines including MDA-MB-231, SUM102, SUM149, MCF-7. All slides were counter stained with DAPI to distinguish nuclear. In SUM102 staining, the positive signal was emitted to red, and other staining in IMEC, MCF-7, MDA-MB-231 and SUM149 cells, positive signal was presented with green. The staining showed that cytokeratin 14 was strongly positive in IMEC cells and SUM102 cell, and lightly positive in SUM149 cells. And cytokeratin 18 positive signals could be detected in IMEC, MCF-7, MDA-MB-231, SUM102 and SUM149 cells. While, cytokeratin 19 positive signal could be found in MCF-7, MDA-MB-231 and SUM149 cells.

2. Localization of delta-N-p63 in breast cancer cell lines:

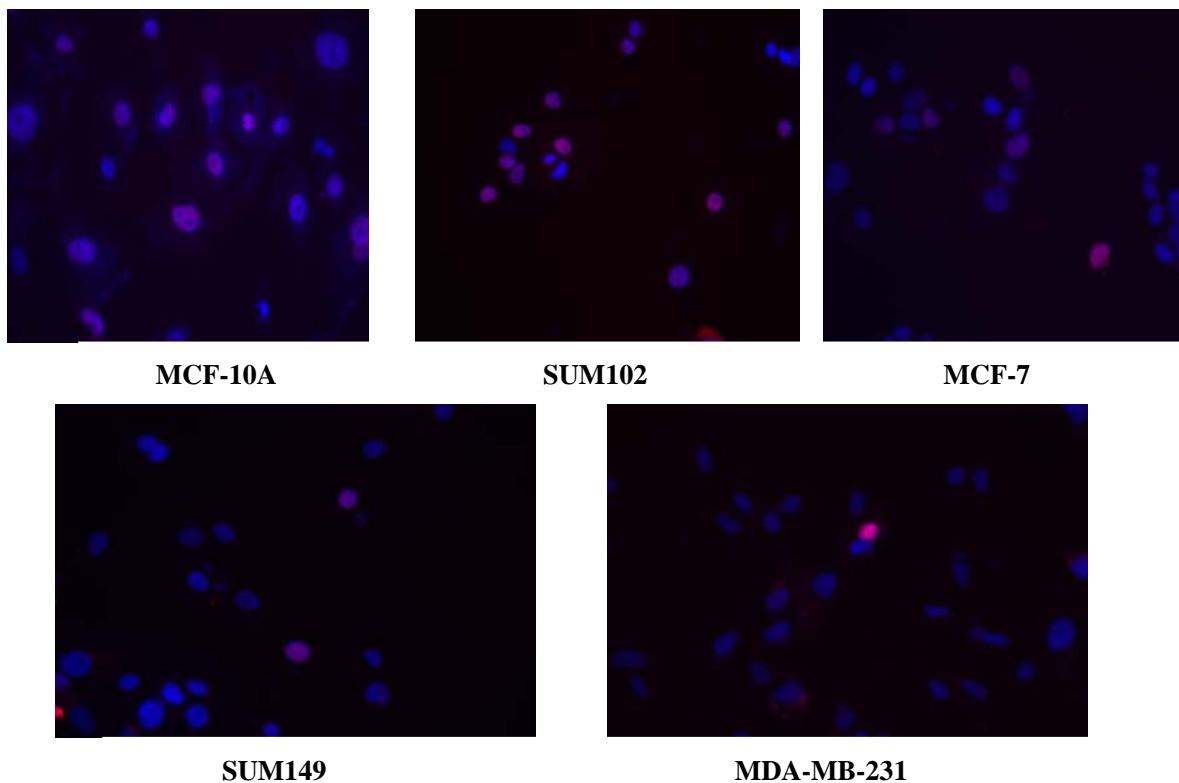
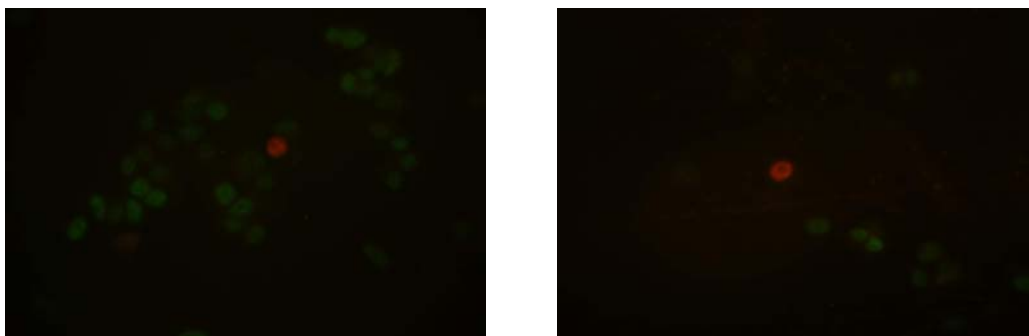
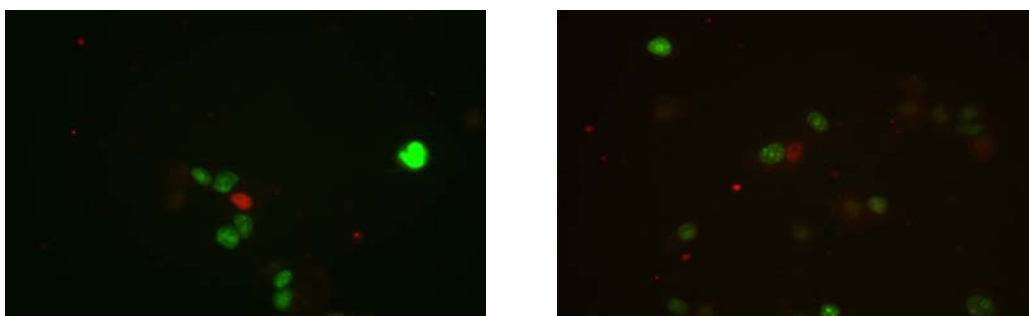


Figure 2: Immunocytofluorescence staining was utilized to detect endogenous expression of delta-N-p63 in breast cancer cells. Pan-p63 (4A4) and delta-N isoform specific primary antibodies were applied to confirm the staining specificity. Secondary antibody was conjugated with Alexfluorescence 594 and all the slides were counter stained with DAPI. The staining clearly showed that delta-N-p63 existed in most tested breast cells including MCF-10A, SUM102, SUM149, MDA-MB-231 and MCF-7 cells, but not in MDA-MB-468 cell.

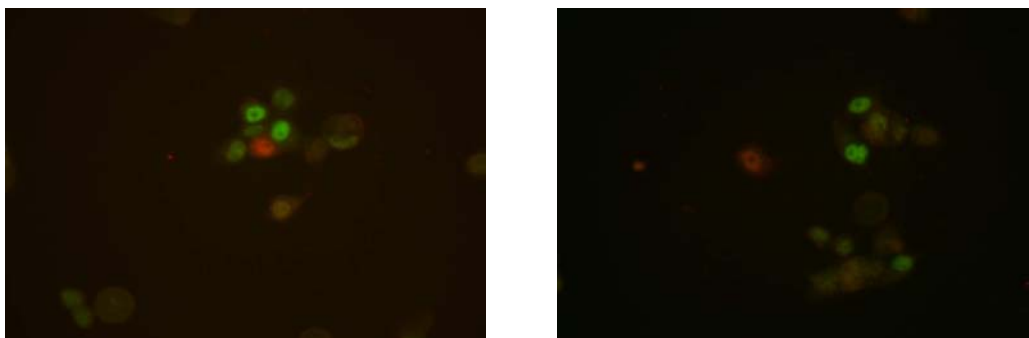
3. Biological character of delta-N-p63 positive cells in MCF-7 cell line:



Estrogen receptor (green) is not co-localized with delta-N-p63 (red) in MCF-7 cell



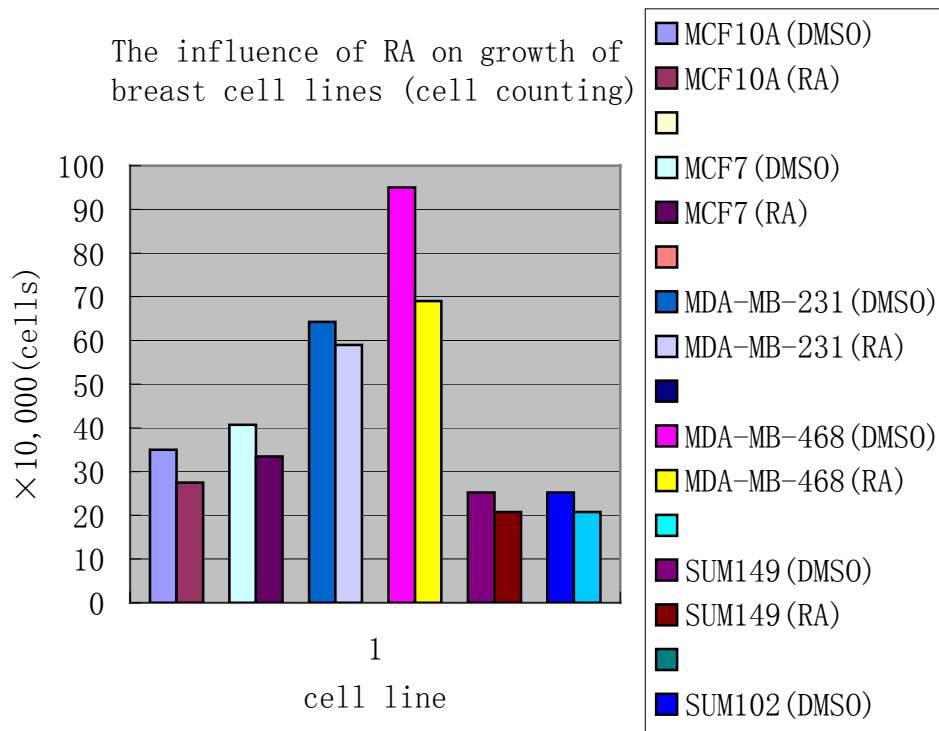
Ki-67 (green) is not co-localized with delta-N-p63 (red) in MCF-7 cell



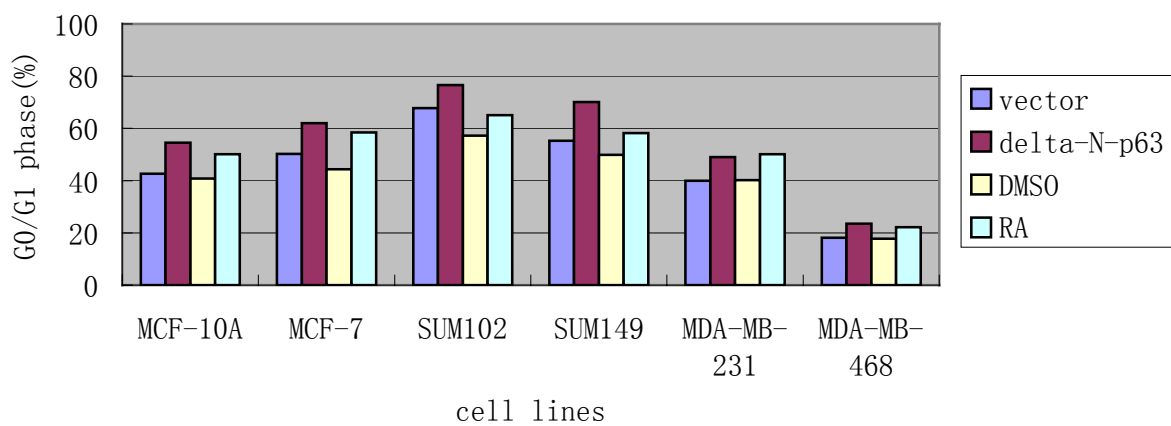
Cyclin D1 is not co-localized with delta-N-p63 (red) in MCF-7 cell

Figure 3. Two-color immunofluorescence staining was applied to analyze the biological character of delta-N-p63 positive MCF-7 cells. The red staining in all pictures are delta-N-p63 positive signal. The staining clearly showed that there is no overlapping between delta-N-p63 positive signal and estrogen receptor, ki-67 and cyclin D1 staining in MCF-7 cells, which implicated that delta-N-p63 positive cells are not end-differentiated and have left proliferative cell cycle.

4. Both retinoic acid treatment and over expression of ectopic delta-N-p63 could inhibit proliferation of breast cancer cells.



Effect of delta-N-p63 and RA treatment on cell cycle of breast cell lines



The influence of overexpression of delta-N-p63 on proliferation of breast cancer cells (cell counting)

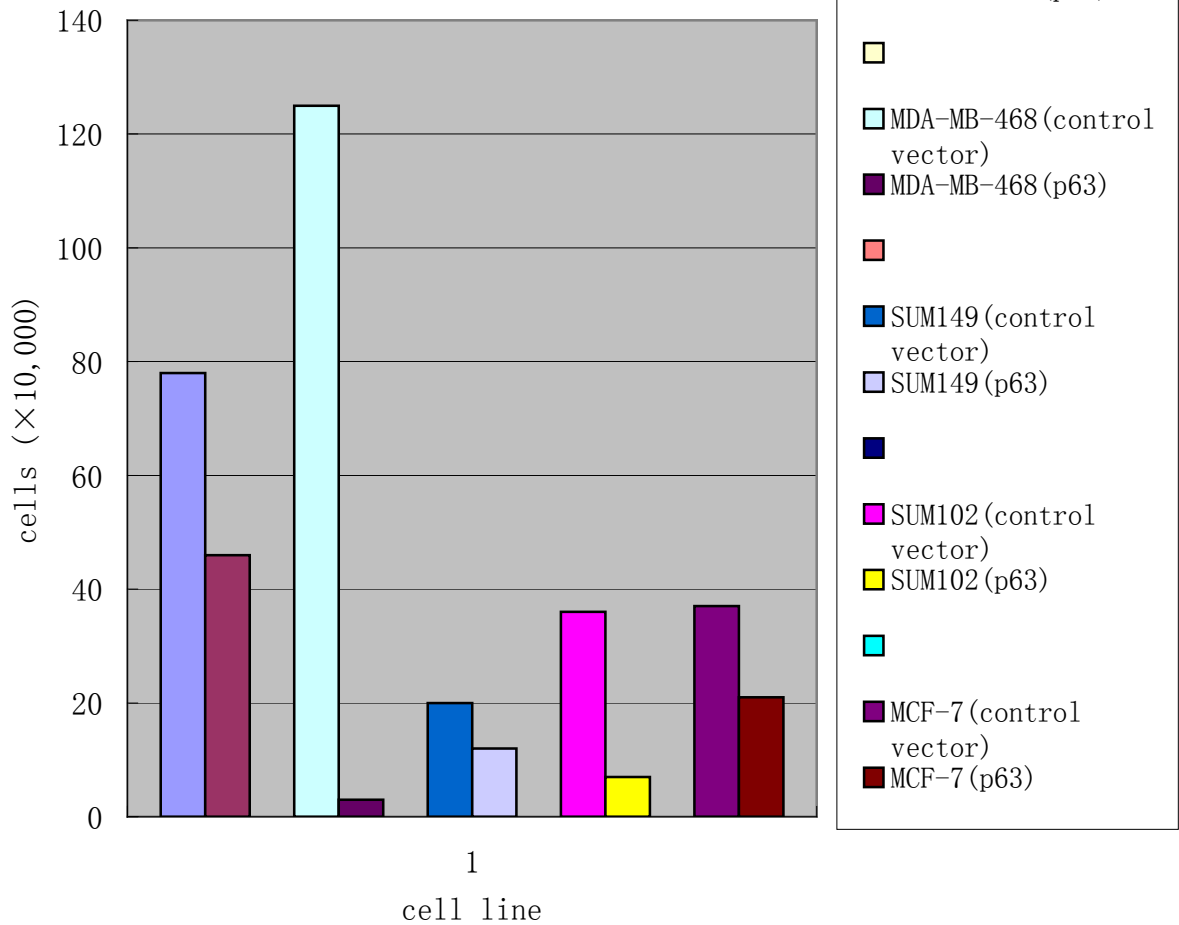
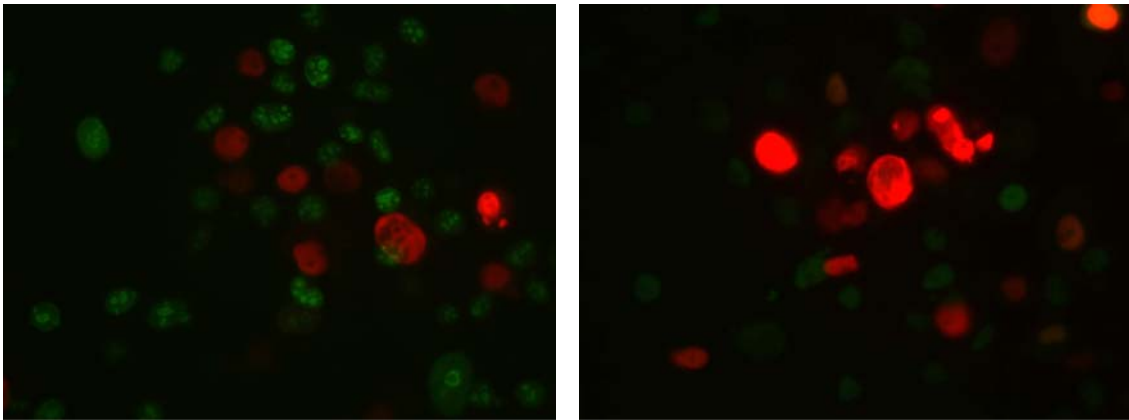


Figure 4. Cell counting and PI-staining flowcytometry assay was applied to analyze the effect of retinoic acid treatment and ectopic delta-N-p63 on proliferation rate and cell cycle of breast cancer cells. The breast cancer cells were treated with 1umol/L RA and 0.01% DMSO as vehicle control, infected with delta-N-p63 and GFP empty vector adenovirus as vehicle control respectively for 72 hours, then cells were collected for cell counting and PI-staining. The cell counting data showed that both RA treatment and over expression of delta-N-p63 could inhibit the proliferation of all tested breast cancer cells significantly. Meanwhile, RA treatment and ectopic delta-N-p63 could cause G1/G0 cell cycle arrest in all examined breast cancer cells.

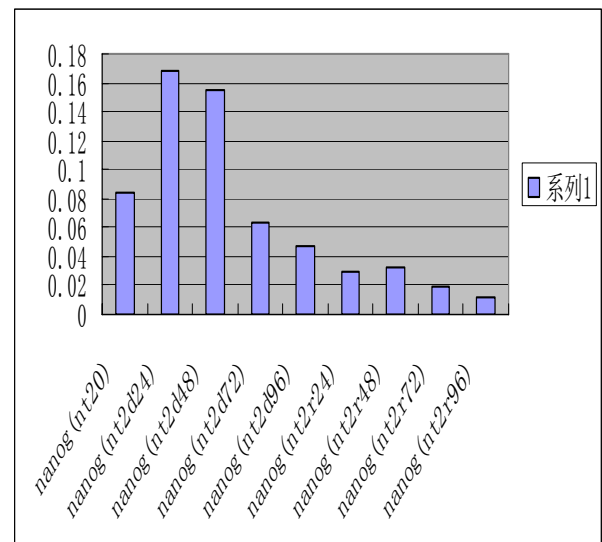
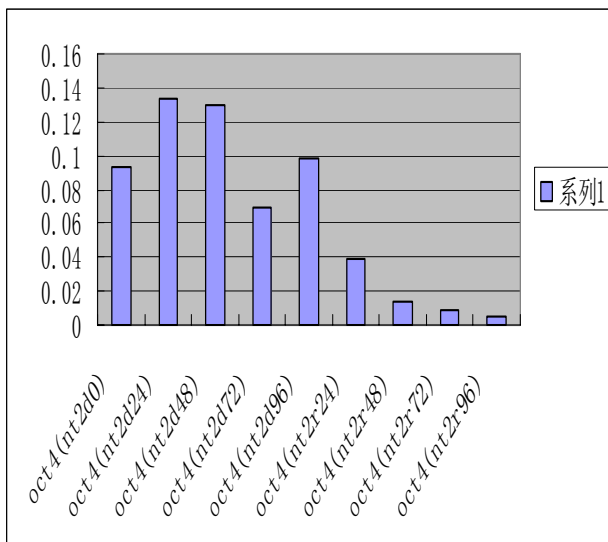
5. Over expression of delta-N-p63 in MCF-7 cells could lead up to cells lose expression of cell cycle marker ki-67 and cyclin D1.

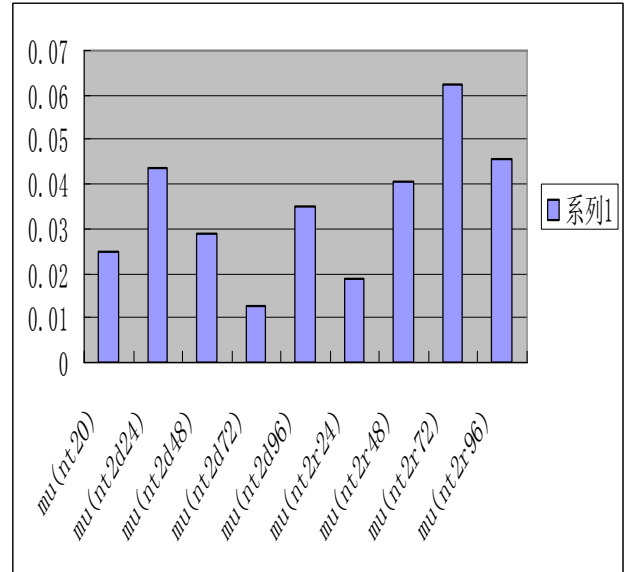
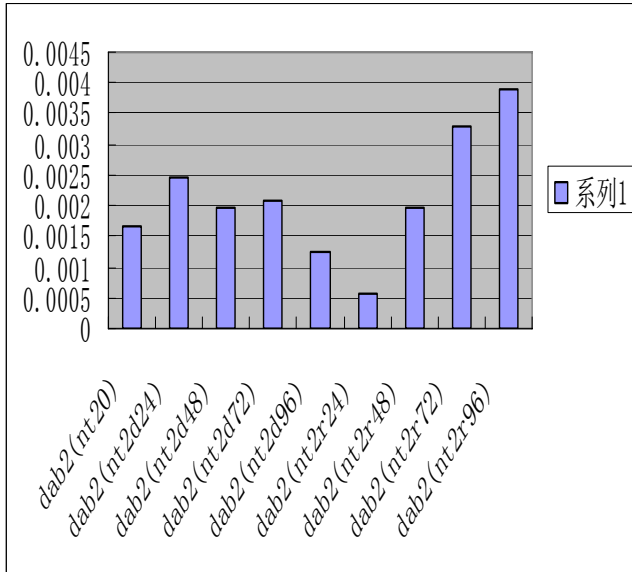


Delta-N-p63(green) positive cells lost expression of ki-67(left, red) and cyclin

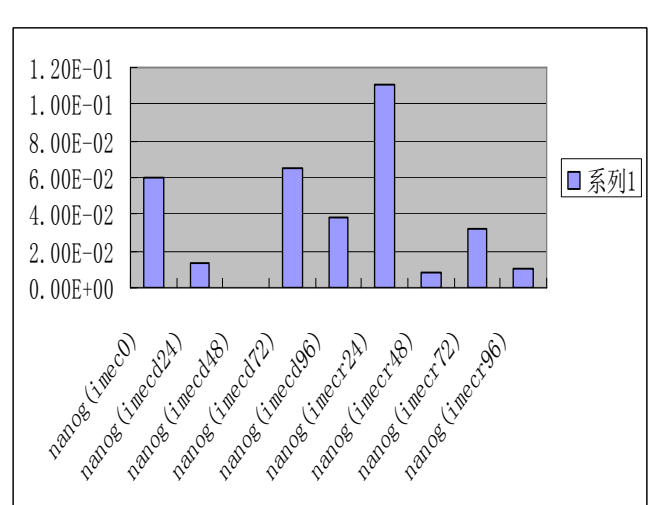
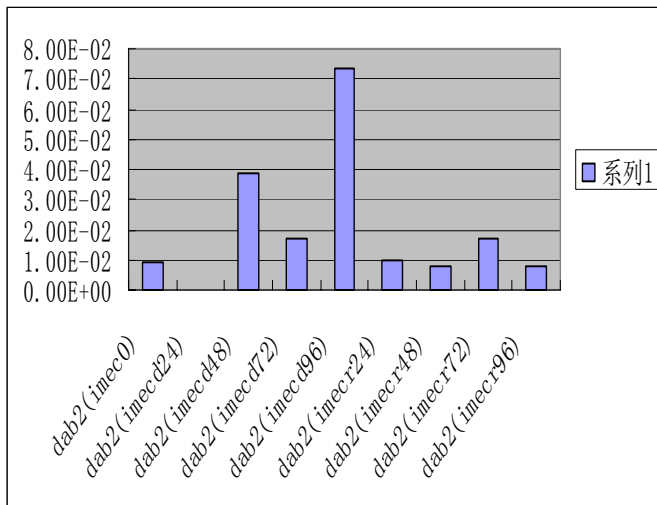
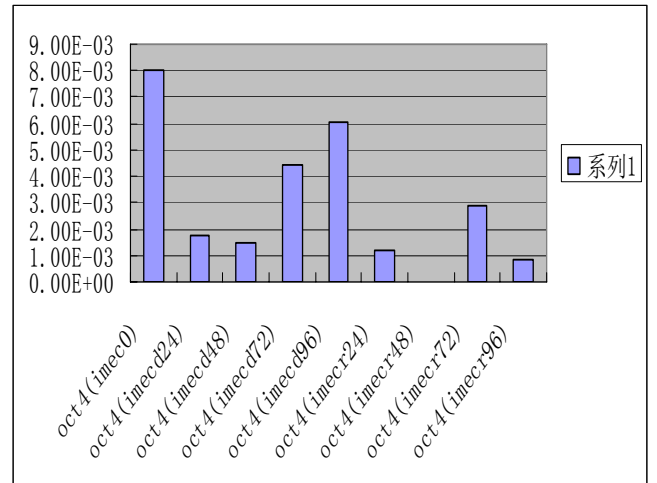
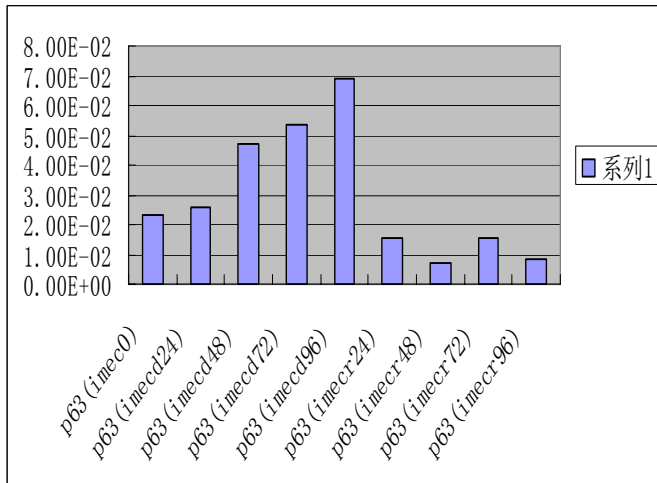
Figure 5. MCF-7 cells were infected with delta-N-p63-alpha and GFP empty vector adenovirus respectively for 72 hours. Then cells were fixed with 3.7% paraformaldehyde for two-color immunofluorescence. All samples were stained with delta-N-p63 and ki-67, cyclin D1 together. The co-staining clearly demonstrated that delta-N-p63 positive cells are more than empty vector group (picture not presented), and there is still no overlapping between delta-N-p63 and ki-67, cyclin D1 staining, which suggested that over expression of ectopic delta-N-p63 could cause cells to lost expression of ki-67 and cyclin D1.

6. The influence of retinoic acid treatment on transcript level of self-renew relative genes

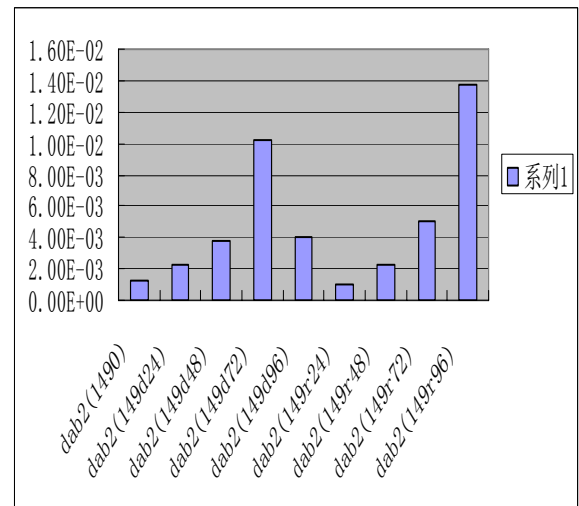
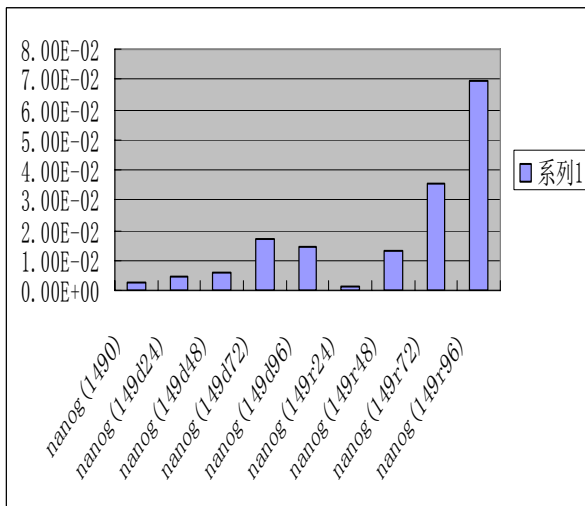
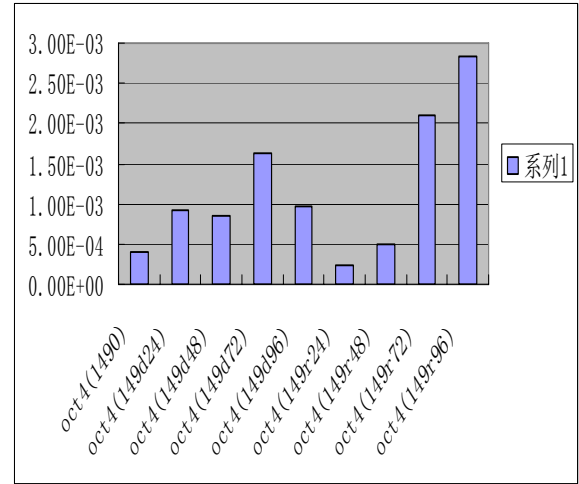
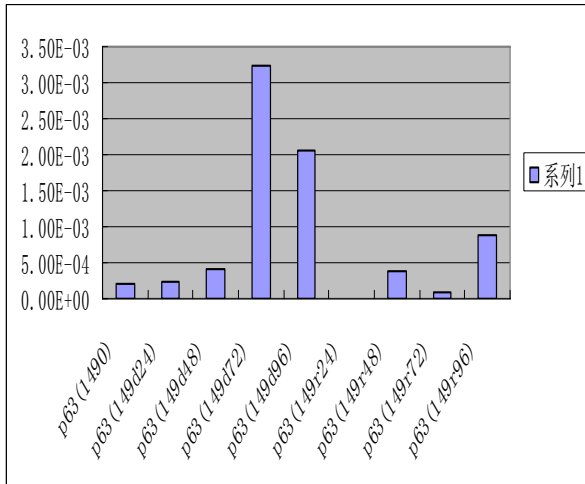




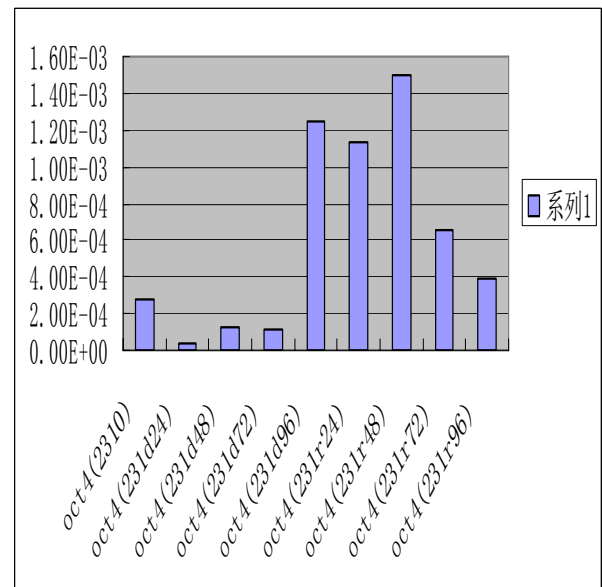
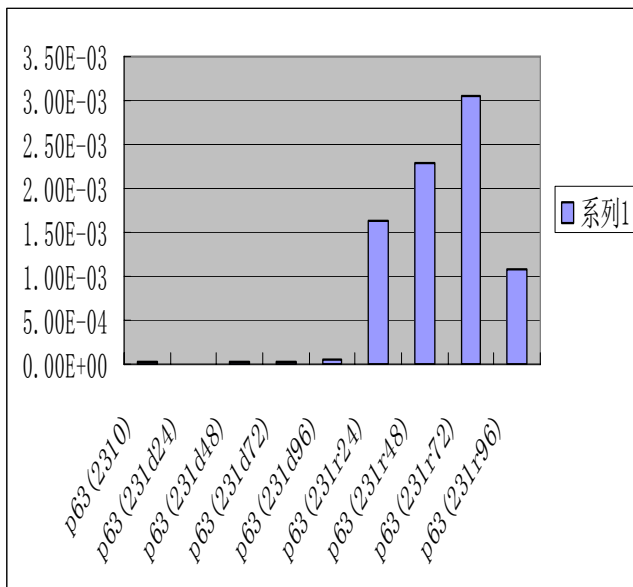
The influence of retinoic acid treatment on transcript level of candidate genes in NT2/D1 cells

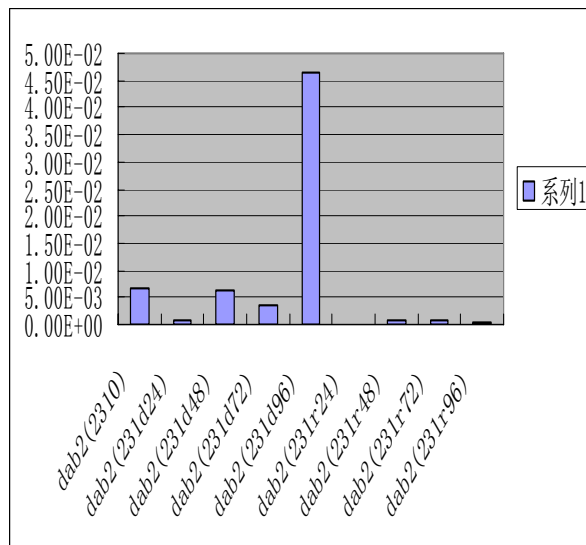
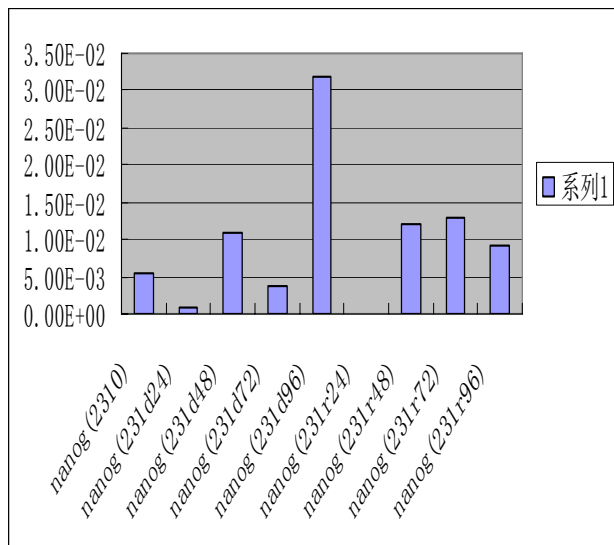


The influence of retinoic acid treatment on transcript level of candidate genes in IMEC cells

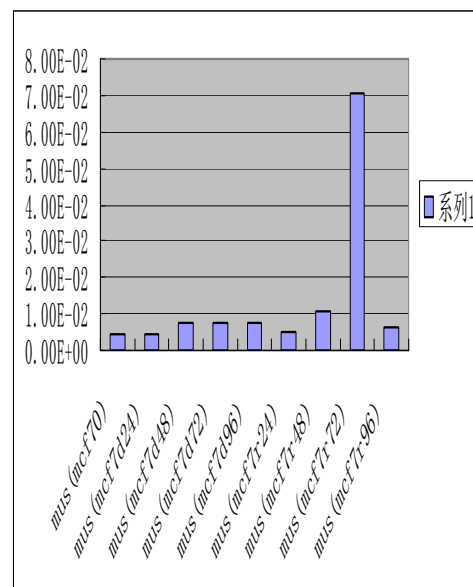
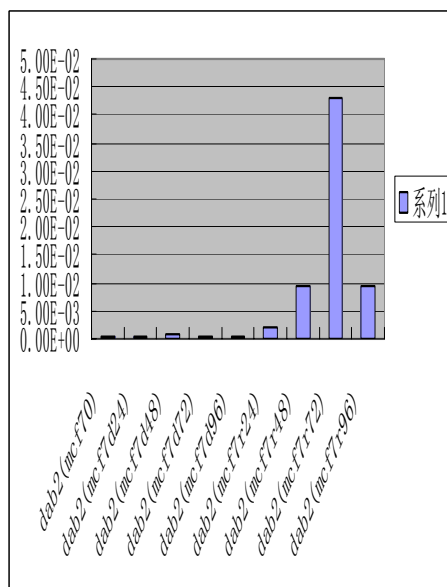
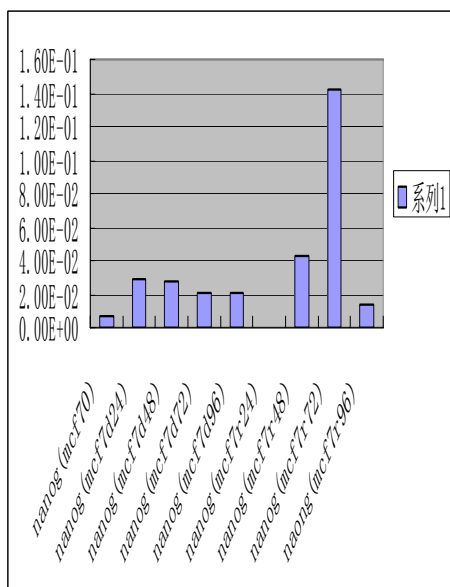
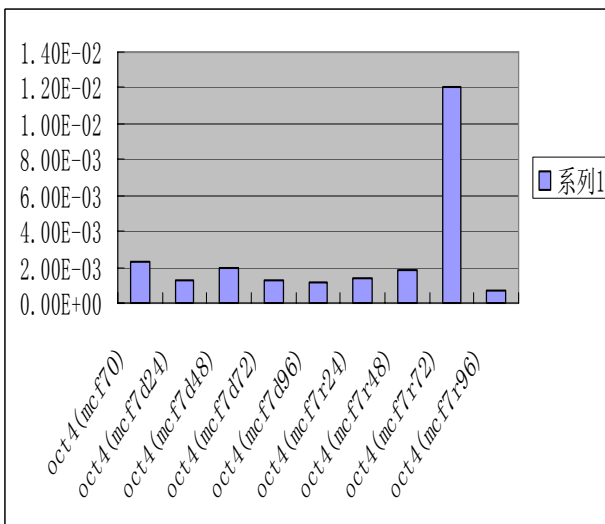
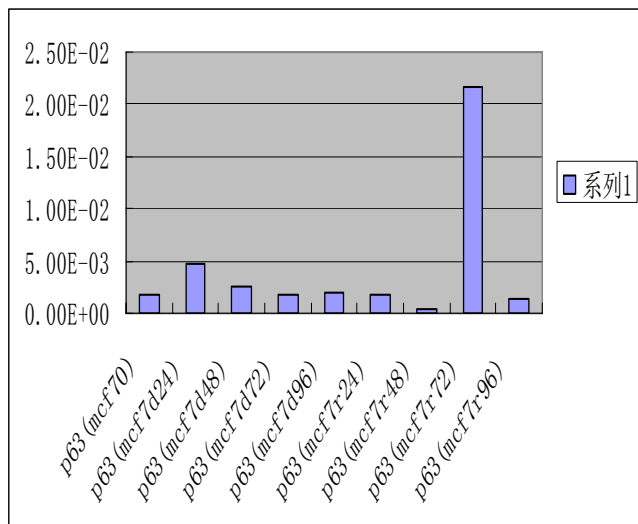


The influence of retinoic acid treatment on transcript level of candidate genes in SUM149 cells

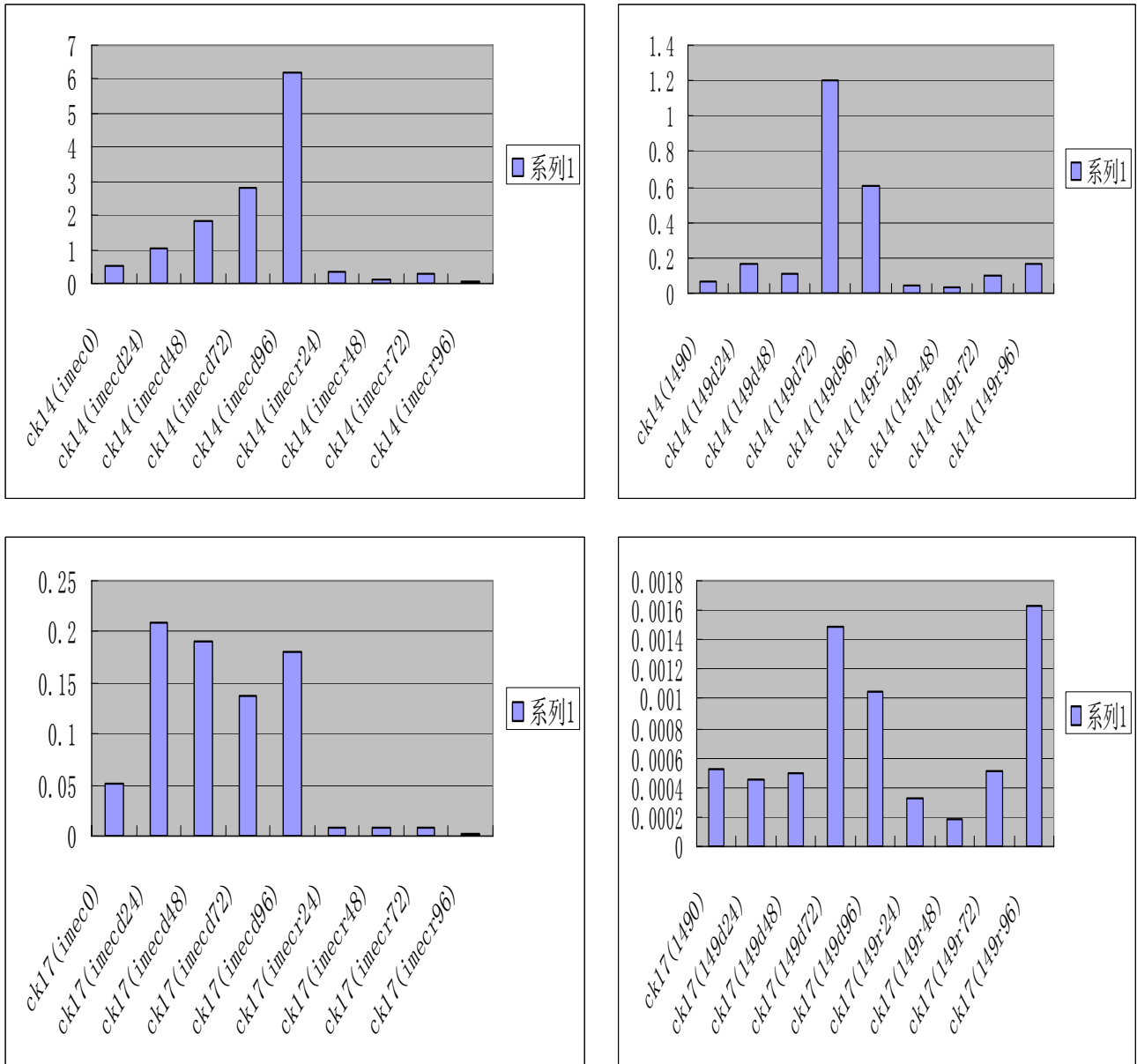




The influence of retinoic acid treatment on transcript level of candidate genes in MDA-MB-231 cells



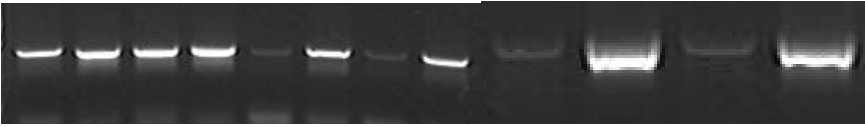
The influence of retinoic acid treatment on transcript level of candidate genes in MCF-7 cells



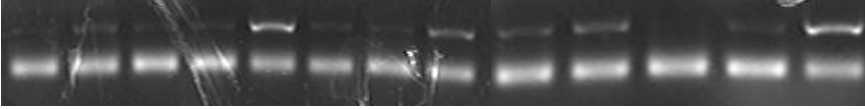
The influence of retinoic acid treatment on transcript level of cytokeratin 14, 17 in IMEC and SUM149 cells

Figure 6. Summary of quantitative PCR analysis of influence of retinoic acid treatment on transcript level of self-renewal relative gene events such as oct3/4, nanog, dab2, cytokeratin 14, 17 in NT2/D1, IMEC, MCF-7, SUM149, MDA-MB-231 cells. All the data have been normalized with GAPGH.

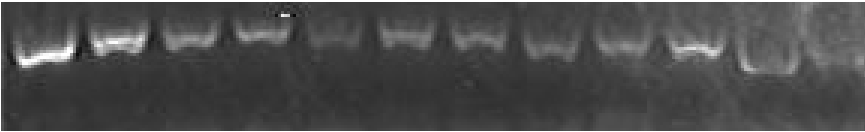
7. Semi-quantitative PCR analysis of influence of ectopic delta-N-p63 on self-renew relative genes in breast cancer cells



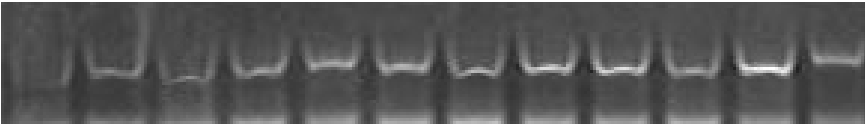
Delta-N-p63



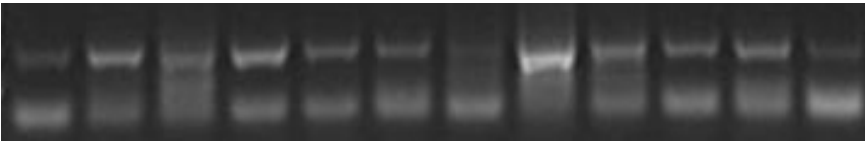
TA-P63



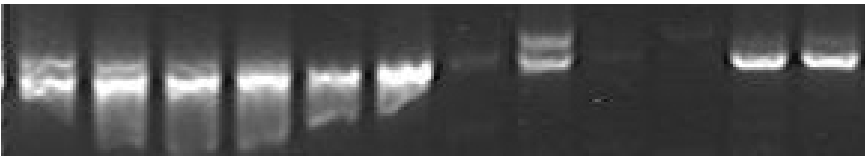
Oct3/4



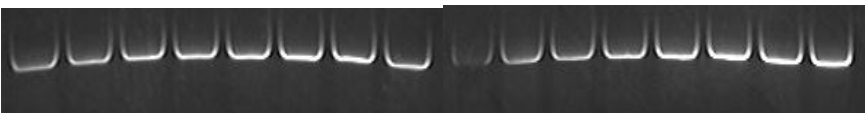
nanog



Sonic hedgehog



Cytokeratin 17



GAPDH

MDA-MB-468C(p63)
MDA-MB-468(vector)
MDA-MB-231(p63)
MDA-MB-231(vector)
MCF-7(p63)
MCF-7(vector)
SUM149(p63)
SUM149(vector)
SUM102(p63)
SUM102(vector)
MCF-10A(p63)

Bullets of accomplishments:

1. Perform preliminary nestin biological function analysis in embryonic carcinoma cell line NT2/D1 with abundant endogenous nestin, and discovered that ectopic delta-N-p63 could block retinoic acid induced differentiation with preservation of transcript level of nestin in NT2/D1 cells.
2. Quantitative PCR analysis of effect of retinoic acid treatment on some self-renew relative gene events in breast cancer cell lines.
3. Localization of breast cancer cell lines with endogenous delta-N-p63.
4. Biological character analysis of delta-N-p63 positive cells in MCF-7 cells with and without over expressed delta-N-p63.
5. Over expression of delta-N-p63 in five different breast cancer lines including MCF-7, SUM102, SUM149, MDA-MB-231, MDA-MB-468 and further examination of effect of delta-N-p63 on proliferation as well as cell cycle.
6. Preliminary semi-quantitative PCR test of the effect of ectopic delta-N-p63 on some self-renew relative gene events in breast cancer cell lines.

Reportable Outcome

The manuscript published in Cancer Research, January 15, 2007.

Hua Li, Pratima Cherukuri, Na Li, Victoria Cowling, Michael Spinella, Michael Cole, Andrew K Godwin, Wendy Wells and James DiRenzo. Nestin is expressed in putative mammary progenitors and is a selective marker of basal epithelial breast tumors. Cancer Research, January 15, 2007, 67(2): 501-510

Conclusions

In this annual report we report that delta-N-p63 could block retinoic acid induced differentiation in embryonic carcinoma cell line NT2/D1, which has abundant endogenous nestin and no measurable endogenous delta-N-p63. In addition, transfection of delta-N-p63 into NT2/D1 cells could preserve transcript level of nestin post RA treatment. All these findings implicated delta-N-p63 could protect cells from differentiation through anti-retinoic acid function. To further analysis biological function of delta-N-p63 and identify genes events correlated with self-renew process mediated by delta-N-p63, we localized breast cancer cell lines with endogenous delta-N-p63. Immunofluorescence staining of pan-p63 and delta-N specific antibodies has confirmed that there is endogenous delta-N-p63 not in estrogen receptor negative SUM102, SUM149, MDA-MB-231 cells, but also MCF-7 cells with luminal epithelial phenotype. Further two-color immunofluorescence analysis shows that delta-N-p63 positive cells are not end-differentiated, estrogen receptor positive cells and lose expression of cell cycle marker, ki-67 and cyclin D1. Over expression of delta-N-p63 in breast cancer lines could lead up to inhabitation of growth rate and G₁/G₀ cell cycle arrest. Moreover, in MCF-7 cells, ectopic delta-N-p63 also could force tumor cells to lose expression of ki-67 and cyclin D1. Quantitative PCR analysis demonstrated that retinoic acid treatment could down-regulate self-renew relative genes such as oct3/4, nanog, dab2 in NT2/D1 and breast cancer cell lines, but the effect of ectopic delta-N-p63 on transcript level of such gene events in breast cancer cell line are more diversified.

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