Role of Neogenin in Regulating Proliferation and Apoptosis in Breast Cancer

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**Keywords**: Neogenin, proliferation, apoptosis, breast cancer.

Abstract. In our former study we have published that the expression of Neogenin was reduced in both breast cancer cells and breast cancer tissues. The aim of this study was to explore the role of Neogenin in regulating proliferation and apoptosis in breast cancer. Firstly, Neogenin expression in MDA-MB-231 cells was up-regulated by using pcDNA3.1-eGFP-Neogenin plasmids. The results of transfection were detected by fluorescent microscope observation, real-time PCR test and western blot. Then the proliferation and apoptosis of MDA-MB-231 cells after Neogenin being up-regulated were tested by using CCK8 assay, flow cytometry assay and TUNEL staining. CCK8 results showed that MDA-MB-231 cells proliferated more quickly after transfected by pcDNA3.1-eGFP-Neogenin while compared with pcDNA3.1-eGFP and MDA-MB-231 groups (P<0.01). Flow cytometry assay showed that there were fewer cells in S phase of cell cycle in pcDNA3.1-eGFP-Neogenin group and more cells in G0/G1 phase while compared with other two groups(P<0.05). TUNEL test showed that the apoptosis rate in pcDNA3.1-eGFP-Neogenin group was significantly higher than other two groups(P<0.05). We found that up-regulating Neogenin expression would inhibit proliferation and promote apoptosis in MDA-MB-231 cells. However, further studies are still needed to explore the mechanism of these variations in proliferation and apoptosis.

Introduction

The metastatic spread of the breast cancer is the most common cause of cancer death in females\cite{1}. Tumorigenesis is a multi-step process and lots of gene related breast cancer have been identified as prognostic markers and new therapeutic targets\cite{2}.

Neogenin is a transmembrane receptor and shares 50% amino-acid identity with the human tumor suppressor molecule deleted in colon cancer (DCC)\cite{3}. As a netrin receptor, it can bind to repulsive guidance molecules (RGM)\cite{4}. It has been proved that Neogenin was involved in cell to cell recognition and cell migration. Recently it has been well accepted that Neogenin is present in tissues where active growth takes place, and overexpression of Neogenin has been observed in a wide variety of human cancers including those of the breast, pancreas, cervix, colon, medulloblastoma and rectum\cite{5}. In our former study we have published that the expression of Neogenin was reduced in both breast cancer cells and breast cancer tissues\cite{6}. However, to date little is known about its function during the procedure of tumorgenisis and
progression of breast cancer. In this study, we up-regulated Neogenin expression in breast cancer cells and detect variation to illustrate its role in proliferation and apoptosis of breast cancer.

**Materials and Methods**

**Cell Culture and Transfection**

MDA-MB-231 cell was from Cell Center of Shanghai Institutes for Biological Sciences (Shanghai, China). pcDNA3.1-eGFP-Neogenin was from addgene(#13031). MDA-MB-231 cells were cultured in growth medium for tumourigenic human breast cells (high glucose DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin). Twenty-four hours before transfection, cells were plated at a density of 10^5 cells/well in 24-well plate and allowed to grow to 80% confluency. Lipofectamine 2000 Reagent (Invitrogen, Van Allen Way Carlsbad, CA, USA) was used for transient transfection according to the manufacturer’s protocol.

**Quantitative Real-time PCR**

Total RNA was extracted from cells using Trizol (Takara, Japan) and cDNA was synthesized using Primerscript RT reagent (Takara, Japan). Relative expression level of Neogenin was normalized to the expression level of GAPDH. The PCR reactions were performed using a 7500 Real-Time PCR System (Applied Biosystems, USA). The primers used in this study were as follows: primers for Neogenin were 5’-ACA TGC TGC ACT GA T CAC CA-3’ and 3’-TCA TAG GTG GGA GGT CCT GG-5’; for GAPDH were 5’-TGA TGA CAT CAA GAA GGT GGT GAA G-3’ and 5’-TCC TTG GAG GCC ATG TGG GCC AT-3’.

**Western Blotting**

The protein was extracted from MDA-MB-231 cells and transferred to polyvinylidene difluoride (PVDF) membranes. Then antibodies were as follows: the goat anti-Neogenin antibody (1:1000, Santa Cruz Biotechnology, California, USA); the horse-radish peroxidase conjugated anti-goat IgG (1:8000, Sigma-Aldrich, Missouri, USA). GAPDH was used as an internal control.

**CCK8 Assay**

The CCK8 assay was carried according to the protocol (7Sea-Cell Couting kit; 7Sea Biotech, China). In general, 10ul CCK8 solution was added to each plate and cells were incubated for 2 h in 37°C. Then the absorbance at 450 nm was measured using a Paradigm Detection Platform (Beckman Coulter, Brea, CA, USA).

**Flow Cytometry**

The cells were digested with trypsin and centrifuged at 1200 rpm for 5 min and washed twice in PBS. Afterwards, 4 ml ice-cold 75% ethanol was added in this solution to fix cells overnight. The centrifuged cells were then added with 500ul PI staining solution and incubated for 30 min. The distribution of cells was analyzed by a FACS Calibur flow cytometer with Cell Quset software (BD Biosciences).
TUNEL Analysis

In Situ Cell Death Detection kit (Roche Applied Science) was used according to the protocol. Briefly, cells were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 25 μg/ml proteinase K for 30 min at 37 °C, and then incubated with 90 μl of labeling solution plus 10 μl of enzyme solution at 37 °C for 2 h. After washed with PBST, the cells were incubated with peroxidase at 37 °C for 30 min. Cell images were acquired by fluorescence microscopy (Axio-skop, Zeiss). The number of TUNEL-positive cells per 1000 cells was counted, and the percentage of apoptotic cells was calculated.

Statistical Analysis

All data were obtained from at least three independent experiments and are presented as mean ± SD. Statistical analysis was performed using one-way ANOVA and student T-test methods. Data were considered significant if p<0.05.

Results

Up-regulation of Neogenin Expression in MDA-MB-231 Cells

In Figure 1A, we observed MDA-MB-231 cells were successfully transfected by pcDNA3.1-eGFP-Neogenin and pcDNA3.1-eGFP. Neogenin mRNA expression was detected by using both RT-PCR and real-time PCR. The results showed Neogenin mRNA was obviously increased in pcDNA3.1-eGFP-Neogenin group while compared with pcDNA3.1-eGFP and MDA-MB-231 cell group(P<0.05)( Figure 1BC).

Figure 1. Expression of Neogenin mRNA in MDA-MB-231 cells after transfected by pcDNA3.1-eGFP-Neogenin.
A. Under fluorescent microscope (200×), we observed that transfection efficiency of pcDNA3.1-eGFP-Neogenin and pcDNA3.1-eGFP was approximately 40~50%. B. Taking GAPDH as control, Neogenin mRNA was increased after transfected with pcDNA3.1-eGFP-Neogenin. C. Real-time PCR results showed that Neogenin mRNA was increased after transfected with pcDNA3.1-eGFP-Neogenin (P< 0.05).

Western blot results showed that Neogenin protein was lowered down after transfected by pcDNA3.1-eGFP-Neogenin while compared with pcDNA3.1-eGFP and MDA-MB-231 cell group (P<0.05), and there was no significant differences between pcDNA3.1-eGFP and MDA-MB-231 cell group (Figure 2).

**Figure 2. Expression of Neogenin protein in MDA-MB-231 cells after transfected by pcDNA3.1-eGFP-Neogenin.**

A and B. Western blot results showed Neogenin protein was increased after transfected with pcDNA3.1-eGFP-Neogenin (P < 0.05).

**Neogenin Inhibit Proliferation of MDA-MB-231**

After inoculating cells from the three groups, which are pcDNA3.1-eGFP-Neogenin group, pcDNA3.1-eGFP group and MDA-MB-231 group, we operated CCK8 tests separately on the second day, the fourth day and the sixth day. CCK8 results showed that on the fourth day the number of cells in pcDNA3.1-eGFP-Neogenin group was 5.53±0.32×10^4/mL, in pcDNA3.1-eGFP was 5.20±0.45×10^4/mL and in MDA-MB-231 was 3.22±0.65×10^4/mL. On the sixth day the number in pcDNA3.1-eGFP-Neogenin group was 6.55±0.13×10^4/mL, in pcDNA3.1-eGFP was 6.34±0.53×10^4/mL and in MDA-MB-231 was 2.77±0.12×10^4/mL (Figure 3).

**Figure 3. Cell proliferation test by CCK8 assay after transfected by pcDNA3.1-eGFP-Neogenin.**
CCK8 results showed that MDA-MB-231 cells proliferated more quickly after transfected by pcDNA3.1-eGFP-Neogenin while compared with pcDNA3.1-eGFP and MDA-MB-231 group (P<0.01).

**Cell Cycle Detection by Flow Cytometry after Up-regulated Neogenin**

Flow cytometry results showed that in pcDNA3.1-eGFP-Neogenin group there were fewer cells in S phase of cell cycle and more cells in G0/G1 phase while compared with other two group(P<0.05).Besides, SPF and PI value was significantly lower than that in other two groups(P<0.05) (Figure 4 and Table 1).

![Flow cytometry results](image)

**Figure 4. Analyze of cell cycle by Flow cytometry after transfected with pcDNA3.1-eGFP-Neogenin.** ($\overline{x} \pm s$, n=3).

Flow cytometry assay showed that there were fewer cells in S phase of cell cycle in pcDNA3.1-eGFP-Neogenin group and more cells in G0/G1 phase while compared with other two group(P<0.05)

**Table 1. Flow cytometry analysis of cell cycle after transfected with pcDNA3.1-eGFP-Neogenin.**

<table>
<thead>
<tr>
<th>Group</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
<th>SPF(%)</th>
<th>PI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>56.72±1.87</td>
<td>18.27±3.57</td>
<td>25.00±1.99</td>
<td>18.27±2.47</td>
<td>43.27±2.46</td>
</tr>
<tr>
<td>pcDNA3.1-eGFP</td>
<td>54.92±2.47</td>
<td>18.42±1.33</td>
<td>26.66±2.37</td>
<td>18.42±3.07</td>
<td>45.08±1.58</td>
</tr>
<tr>
<td>pcDNA3.1-eGFP-Neogenin</td>
<td>70.21±1.96*</td>
<td>9.32±1.87*</td>
<td>20.47±3.31</td>
<td>9.32±1.97*</td>
<td>29.79±1.63*</td>
</tr>
</tbody>
</table>

**Apoptosis Detection by TUNEL after Up-regulated Neogenin**

TUNEL tests showed that there were more apoptosis cells in pcDNA3.1-eGFP-Neogenin group while compared with that in pcDNA3.1-eGFP and MDA-MB-231 group. By counting and calculating, we found that the apoptosis rate in pcDNA3.1-eGFP-Neogenin group was significantly higher than other two groups(P<0.05)(Figure 5).
Figure 5 Analyze of apoptosis rate by TUNEL staining of cells after transfected with pcDNA3.1-eGFP-Neogenin.

The apoptosis rate in MDA-MB-231 group was 9.97±0.13%, in pcDNA3.1-eGFP was 9.65±0.09% and in pcDNA3.1-eGFP-Neogenin was 27.01±1.89%. The apoptosis rate in pcDNA3.1-eGFP-Neogenin group was significantly higher than other two groups (P<0.05).

Discussion

Neogenin is a multi-functional receptor that regulates many diverse developmental processes, including signal transduction, cell migration, tissue development and differentiation, angiogenesis, neural tube formation and apoptosis[3, 7, 8]. Neogenin mRNA and protein are widely expressed in both embryo and adult tissues during development. Evidences showed that Neogenin is a multi-functional receptor regulating many diverse developmental processes, including signal transduction, cell migration, tissue development and differentiation, angiogenesis, neural tube formation and cell apoptosis[9]. Our previous study showed that the expression level of Neogenin was lower in breast cancer tissues as compared to their matched adjacent non-cancerous tissues, and was correlated to the pathological grade of breast cancer[6]. So in this study we focused on the role of Neogenin in proliferation and apoptosis in breast cancer.

In our previous study, we have evaluated the expression level of Neogenin in different cell lines and found that the lowest level of Neogenin was in MDA-MB-231 cells. So in this study we chose MDA-MB-231 and up-regulated Neogenin expression in these cells. By using CCK8 tests we found that proliferation was inhibited after Neogenin being up-regulated and the differences were especially obvious from the fourth day to the sixth day (P<0.05). The role of Neogenin in proliferation was also reported in several cancers. In addition, by flow cytometry, we observed that up-regulating Neogenin will promote cell cycle arrest in MDA-MB-231 cells. Interestingly, in gastric cancer Neogenin ablation decreased proliferation and migration, whereas its over-expression reversed these effects[10]. We inferred that maybe Neogenin function differently in various cancers by respective mechanism.

By TUNEL assay we observed that apoptosis was promoted after Neogenin being up-regulated. The mechanism of Neogenin in inhibiting apoptosis of MDA-MB-231 cells was not quite clear now. In several studies[11], it has been inferred that Neogenin is a multifunctional receptor which could bind to several ligands and deliver signals to modulate...
cell activity. Besides, Neogenin could also function as dependent receptor which means that Neogenin can induce apoptosis of certain types of cells when its ligands are absent. It is reported that alteration of expression in the dependent receptors such as DCC, UNC5H, Neogenin[9] and their ligands, netrin-1[12] and RGMa[13] would cause loss of pro-apoptotic activity and even lead to tumorigenesis[14].

Conclusion

In this study we successfully up-regulated Neogenin expression in MDA-MB-231 cells. After that we further studied the proliferation and apoptosis changes in MDA-MB-231 cells. We found that up-regulated Neogenin expression would inhibit proliferation and promote apoptosis in MDA-MB-231 cells. However, further studies are still needed to explore the mechanism of these variations in proliferation and apoptosis.

References

