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NBR2/miR-561-5p/DLC1 axis inhibited the development of multiple myeloma by activating the AMPK/mTOR pathway to repress glycolysis

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Long non-coding RNA NBR2 exerts a tumor-suppressive effect in a variety of cancers, but its role in multiple myeloma (MM) is unclear. This article will elucidate the role of NBR2 in MM. The expressions of NBR2, miR-561-5p, and deleted in liver cancer 1 (DLC1) in MM cell lines were determined by quantitative real time polymerase chain reaction (qRT-PCR). The regulatory relationship of the NBR2/miR-561-5p/DLC1 axis was predicted by bioinformatics and confirmed via a dual-luciferase reporter assay. The effect of NBR2 on the biological behavior of MM cells was verified by loss- and gain-of-function experiments (cell counting kit-8, colony formation, flow cytometry, extracellular acidification rate, and lactate production measurement). The effects of the NBR2/miR-561-5p axis on the biological behavior of MM cells, the activation of the AMPK/mTOR signaling pathway (western blot), and DLC1 expression (western blot) were verified by rescue experiments. The upregulation of NBR2 in MM cell lines induced a decrease in the viability, proliferation capacity, glycolysis, and lactic acid production, and an increase in apoptosis of MM cells. NBR2 regulated the biological behavior of MM cells and the activation of the AMPK/mTOR signaling pathway by targeting miR-561-5p. DLC1 was the target gene of miR-561-5p and the protein expression of DLC1 was regulated by the NBR2/miR-561-5p axis. Collectively, NBR2/miR-561-5p/DLC1 axis inhibits the development of MM by activating the AMPK/mTOR pathway to repress glycolysis.

Key words: miR-561-5p, NBR2, deleted in liver cancer 1, multiple myeloma

Multiple myeloma (MM) is the second most prevalent tumor in the blood and is a malignant disease characterized by malignant plasma cells that accumulate in the bone marrow and produce monoclonal proteins in the blood or urine [1]. It can cause impaired renal function, anemia, osteolysis lesions, etc. [2]. The current treatments for MM mainly include proteasome inhibitors, monoclonal antibodies, histone deacetylase inhibitors, alkyl drugs, immunomodulators, and so on [3]. These therapeutic strategies have relieved the condition of MM patients, but some patients after treatment will still relapse [4]. Therefore, it is urgent to find new treatments to prolong the survival period of MM patients.

The occurrence and development of MM are related to complex molecular mechanisms. In recent years, the role of long non-coding RNA (lncRNA) in MM has received extensive attention, which is due to the participation of lncRNA in epigenetic regulation, transcription regulation, and post-transcriptional regulation, as well as the important effect that lncRNA exerts in carcinogenesis and tumor suppression [5]. In addition, some reports claim that MM patients can be stratified by the level of lncRNAs expressions, thereby improving the prognosis of MM [6, 7]. The strategy based on lncRNA-targeted therapy for MM is in full swing. Amodio and his colleagues used LNA-gapmeR antisense oligonucleotides to target MALAT1, which can inhibit the proliferation of MM cells and promote their apoptosis *in vivo* and *in vitro* [8]. Although there are many members of the lncRNA family, only a few are related to the prognosis of MM. As such, it is necessary to find new lncRNAs related to MM. A study has underlined that lncRNAs that are dysregulated in other types of cancers have a high probability of being dysregulated in MM as well [9]. LncRNA NBR2 is lower-expressed in several other cancers such as osteosarcoma [10], hepatocellular carcinoma [11], colorectal cancer [12], and thyroid cancer [13]. Nevertheless, its role in MM has not been expounded yet. Through previous experiments, we found that the level of NBR2 was downregulated in MM. Accordingly, we speculated that NBR2 may be involved in regulating the progress of MM.

LncRNAs can be used as competitive endogenous RNAs (ceRNAs) to participate in the regulation of cancer, and play an important role in homeostasis regulation and tumorigen-

esis [14, 15]. Under normal circumstances, lncRNAs with complementary binding regions with microRNAs (miRNAs) can act as the sponges of miRNA, thereby eliminating or reducing the inhibitory effects of miRNAs on their target genes. At present, multiple lncRNA-miRNA-mRNA regulatory axis have been found in MM [16, 17]. However, it is unclear whether NBR2 is involved in the progress of MM through the mechanism of ceRNA. Therefore, this article will interpret the role of NBR2 in MM from the perspective of ceRNA.

Materials and methods

Cell culture. Normal plasma cell line nPCs (BNCC101671, BNBIO, China) and MM cell lines U266B1 (ZK1901-XR, bzwzw, China), H929 (ZK1587-XR, bzwzw, China), MM1.S (ZK1456-XR, bzwzw, China), and KMS11 (ZKCC-X1953, bzwzw, China) were used in this study. All cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (72400120, Gibco, USA) containing 10% fetal bovine serum (10091, Thermo Fisher, USA) and 1% penicillin-streptomycin (15140-122, Thermo Fisher, USA). The culture condition was at 37 °C with 5% CO_2 (Forma Steri-Cycle, Thermo Scientific, USA).

qRT-PCR. The RNA from cells was extracted by Total RNA Extraction Kit (R1200, Solarbio, China). A one-step method was used for reverse transcription and qPCR of extracted RNA. The kits used included fastking one-step qRT-PCR kit (FP313, Tiangen, China) and one step miRNAs qRT-PCR kit (aomd-q020, Genecooeia, USA). The internal control used in the process of qRT-PCR included glucose dehydrogenase-3-phosphate dehydrogenase (GAPDH) and U6. The primers are listed in Table 1. The main instrument used in the process of qRT-PCR was the Real-Time PCR system (7500Fast, ABI, USA), and the calculation was performed using the $2^{-\Delta\Delta Ct}$ method [18].

Transfection. The NBR2 overexpression vector was constructed by cloning the full-length NBR2 sequence into pcDNA3.1+vector (V87020, Thermo Fisher, USA). NBR2 specific small interfering RNA (siNBR2, siG180611025344-1-5), negative control (NC, siN0000001-1-5), miR-561-5p mimic (M, miR10022706-1-5), mimic control (MC, miR1N0000001-1-5), inhibitor (I, miR20022706-1-5), and inhibitor control (IC, miR2N0000001-1-5) were purchased from Ribobio company (China). Lipofectamine™ RNAiMAX (13778100, Thermo Fisher, USA) was applied for transfection. 24 hours after transfection, the success of transfection was determined by the results of qRT-PCR.

Table 1. Primers used in this study.

Primers for PCR	List of oligonucleotide sequences 5'--> 3'
LncRNA NBR2 Forward	GGAGGTCTCCAGTTTCGGTA
LncRNA NBR2 Reverse	TTGATGTGTGCTTCCTGGG
miR-561-5p Forward	ATCAAGGATCTTAAAC
Universal Reverse	GTGCAGGGTCCGAGGT
GAPDH Forward	CAATGACCCCTTCATTGACC
GAPDH Reverse	TTGATTTTGGAGGGATCTCG
U6 Forward	GCTTCGGCAGCACATATACTAAAAT
U6 Reverse	CGCTTCACGAATTTGCGTGTCAT

Cell processing. NBR2 overexpression vector was transfected into U266B1 cells and siNBR2 was transfected into H929 cells to observe the effect of NBR2 on MM cells.

Furthermore, the NBR2 overexpression vector and miR-561-5p mimic were co-transfected into U266B1 cells, while siNBR2 and miR-561-5p inhibitor were co-transfected into H929 cells to observe the effects of NBR2/miR-561-5p on MM cells.

Cell viability test. The viability of U266B1 cells and H929 cells at 24, 48, and 72 hours after transfection was assessed by cell counting kit-8 (CCK-8) assay kit (M4839, AbMole, China). In short, 10 µl of CCK-8 solution was incubated with the treated cells for 2 h. The absorbance at 450 nm was measured with a microplate reader (SpectraMax5, Molecular Devices, USA).

Colony formation assay. 200 transfected cells were cultured in 6-well plates with a complete medium for 10 days. After 10 days, the supernatant was discarded and 4% paraformaldehyde (E672002, Sangon, China) was applied to fix the cells for 15 min. After the cells were stained with 0.1% crystal violet (C0121, Beyotime, China) for 20 min, the number of cell clones was observed under a microscope (BX53M, Olympus, Japan).

Apoptosis detection. Annexin V-FITC/Propidium Iodide apoptosis detection kit (P-CA-201, Procell, China) was applied to evaluate the apoptosis of cells. Cells (5×10^5) were resuspended in 500 µl diluted 1× Annexin V Binding Buffer and then stained with 5 µl of Annexin V-FITC and 5 µl of propidium iodide at room temperature for 15 min in the dark. The main instrument used in this process of flow cytometry was a flow cytometer (DxFLEX, Beckman, USA).

Measurement of extracellular acidification rate (ECAR). The analysis of glycolysis was based on the research of Zhang et al. [19]. The transfected cells (40,000) were inoculated into the XF24 cell culture plate (100867-100, Agilent, USA) and cultured for 24 h. Next, the entire culture plate was centrifuged at 300× g for 1 min, and then the Seahorse XF RPMI medium (103576-100, Agilent, USA) was replaced with an XF analysis medium (supplemented with 5 mM glucose). After the cells were equilibrated under normoxic conditions, the ECAR value was recorded with the XF24 analyzer (Agilent, USA).

Measurement of lactate. The measurement of lactate production referred to the research of Zhang et al. [19]. A Lactate Colorimetric/Fluorometric Assay Kit (#K607, Biovision, USA) was used to determine the production of lactate. In short, the transfected cells were seeded into a

12-well plate at the density of 50,000 cells/well. After the cells adhered to the wall, a fresh medium was added to the plate and incubated for 1 h. Then the content of lactate in each well was evaluated with a fluorescence reader (ELx808, Agilent, USA) and the number of cells was counted at the same time.

Dual-luciferase reporter assay. starBase (http://starbase.sysu.edu.cn/) and Target Scan (http://www.targetscan.org/vert_72/) were applied to predict the relationship between miR-561-5p and NBR2/deleted in liver cancer 1 (DLC1)/RBL1. Then, we amplified the binding sequences of miR-561-5p and NBR2/DLC1/RBL1 and cloned them into the psiCHECK-2 vector (TB329, Promega, USA). These sequences included wild type (WT) and mutant type (MUT) as follows: NBR2-WT (AGCCUCCAGAAAAAUCCUUGAA), NBR2-MUT (AGCCUCCAGAAAACUCGUUGAA), RBL1-WT (UUUUUUAAUGUUUGCUCCUUGAG), RBL1-MUT (UUUUUUAAUGUUUGCUCCAUCAG), DLC1-WT (UAGAACUUUUGCCAGUCCUUGAA), and DLC1-MUT (UAGAACUUUUGCCAGUUCGAGAA). Each vector carrying the above sequence and miR-561-5p mimic/mimic control was co-transfected into cells. The luciferase activity of cells in each group was finally detected by Dual-Luciferase Reporter Assay System (E1910, Promega, USA).

Western blot. Western blot was performed according to the guidelines of Alegria-Schaffer et al. [20]. Simply put, the protein in the cells was extracted and quantified, and then the sample volume was calculated. The protein was denatured, loaded with electrophoresis, and then transferred to the membrane with a current of 250 mA. After being transferred and blocked, the membrane was incubated first with the primary antibody at 4 °C and then with the secondary antibody after washing the membrane on the next day in sequence. Finally, the protein band analyses were carried out. The primary antibodies used in this study were as follows: anti-DLC1 (1:1000, 171 kDa, ab126257, Abcam, UK), anti-phospho-AMPK (p-AMPK, 1:1000, 62 kDa, #50081, Cell Signaling Technology, USA), anti-AMPK (1:1000, 62 kDa, #2532, Cell Signaling Technology, USA), p-mTOR (1:1000, 289 kDa, #5536, Cell Signaling Technology, USA), anti-mTOR (1:1000, 289 kDa, #2972, Cell Signaling Technology, USA), and GAPDH (1:10000, 36 kDa, ab8245, Abcam, UK) antibodies; and the secondary antibodies were goat anti-rabbit IgG (1:5000, ab6721, Abcam, UK) and goat anti-mouse IgG (1:5000, ab205719, Abcam, UK). GAPDH was used as an endogenous normalization control.

Statistical analyses. Data were analyzed by Graph Prism v8.0 (GraphPad Software, USA) and presented as mean ± standard deviation. The correlation between NBR2 and miR-561-5p/DLC1 or DLC1 and miR-561-5p was analyzed by Pearson's correlation test. Differences among multiple groups were analyzed by one-way analysis of variance. A p-value <0.05 was accepted to be statistically significant.

Results

NBR2 was lower-expressed in MM cells. Compared with human normal plasma cell line nPCs, the level of NBR2 in the MM cell line was significantly reduced (Figure 1, p<0.001). Since the expression of NBR2 was relatively higher in H929 cells and relatively lower in U266B1 cells, these two cell lines were selected for the subsequent experiments.

NBR2 modulated the biological functions of MM cells. In order to determine the effect of NBR2 on MM cells, we overexpressed NBR2 in U266B1 cells and silenced NBR2 in H929 cells (Figure 2A, 2B, p<0.001). The viability of U266B1 cells was inhibited after treatment with NBR2 overexpression for 48 and 72 h (Figure 2C, p<0.05), while, the viability of H929 cells was enhanced after treatment with NBR2 silencing for 24, 48, and 72 h (Figure 2D, p<0.05), as compared with the NC or siNC group. Meanwhile, NBR2 overexpression inhibited clone formation (Figures 2E, 2F, p<0.01), promoted the apoptosis (Figures 2I, 2J, p<0.001), and repressed ECAR (Figure 2M, p<0.001), and lactate production in U266B1 cells (Figure 2O, p<0.001), while siNBR2 exerted the opposite effects on H929 cells (Figures 2G, 2H, 2K, 2L, 2N, 2P, p<0.01).

miR-561-5p was the target miRNA of NBR2. The binding sites of NBR2 and miR-561-5p are shown in Figure 3A. It was observed in both U266B1 and H929 cells that miR-561-5p mimic or inhibitor could reduce or increase the luciferase activity of cells in the NBR2-WT group, but did not affect the luciferase activity of those in the NBR2-MUT group (Figures 3B, 3C, p<0.001). As shown in Figure 3D–E, the transfection efficiency of miR-561-5p mimic and inhibitor was detected, miR-561-5p expression was increased after U266B1 cells were transfected with miR-561-5p mimic,

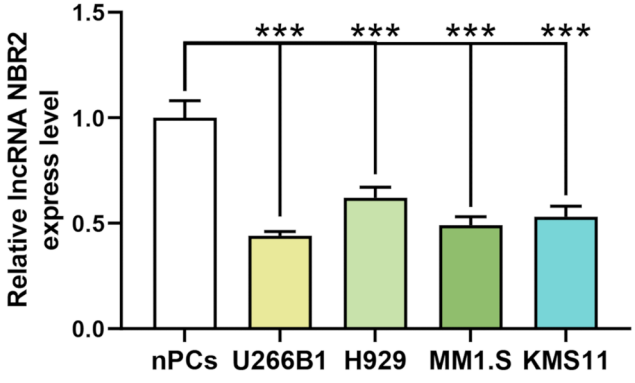

Figure 1. The lower expression of lncRNA NBR2 in MM cells. The expression of lncRNA NBR2 in human normal plasma cell line nPCs and MM cell lines (U266B1, H929, MM1.S, KMS11) was detected by qRT-PCR. GAPDH was used as an internal control. Quantified values were presented as mean ± standard deviation of at least three independent experiments. ***p<0.001 vs. nPCs group. Abbreviations: lncRNA-long non-coding RNA; MM-multiple myeloma; qRT-PCR-quantitative real time polymerase chain reaction; GAPDH-glyceraldehyde-3-phosphate dehydrogenase

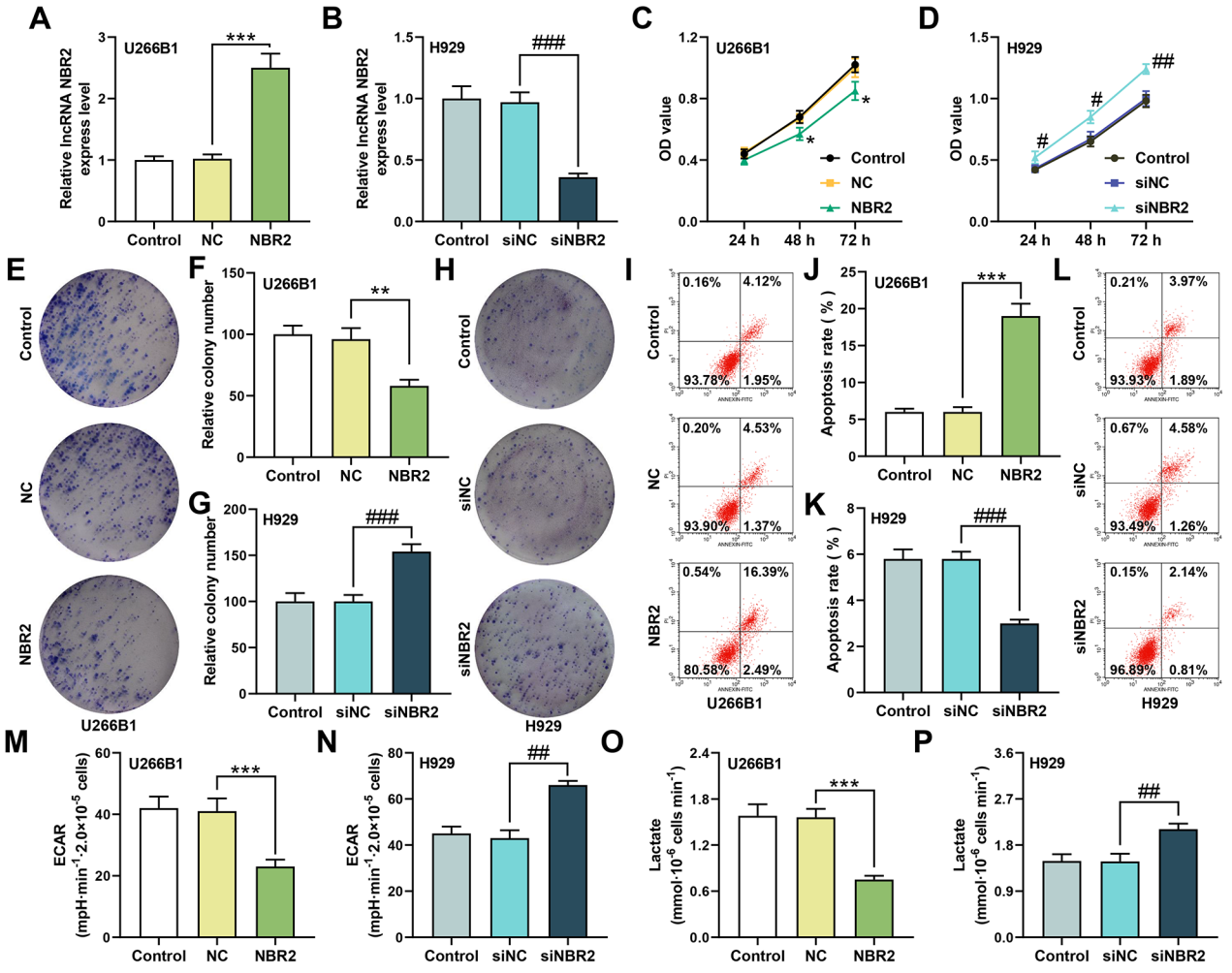

Figure 2. LncRNA NBR2 regulated the viability, proliferation, apoptosis, ECAR, and lactate production of MM cells. A, B) qRT-PCR evaluated the transfection efficiency of NBR2 overexpression vector and siNBR2 in MM cell lines U266B1 and H929. GAPDH was used as an internal control. C, D) The viability of MM cells transfected with NBR2 overexpression vector and siNBR2 was determined by the CCK-8 assay. E–H) The effect of NBR2 overexpression or siNBR2 on the proliferation of MM cells was evaluated by the clone formation experiment. I–L) The effect of NBR2 overexpression or siNBR2 on the apoptosis of MM cells was evaluated by flow cytometry. M, N) ECAR in MM cells was detected by the Seahorse-XF24 analyzer. O, P) The production of lactate in MM cells was evaluated using the lactate assay kit. Quantified values were presented as mean ± standard deviation of at least three independent experiments. *p<0.05, **p<0.01, ***p<0.001 vs. NC group. #p<0.05, ##p<0.01, ###p<0.001 vs. siNC group. Abbreviations: lncRNA-long non-coding RNA; MM-multiple myeloma; ECAR-extracellular acidification rate; qRT-PCR-quantitative real time polymerase chain reaction; GAPDH-glyceraldehyde-3-phosphate dehydrogenase; siNBR2-NBR2 specific small interfering RNA; CCK-8-cell counting kit-8

while miR-561-5p expression was decreased after H929 cells were transfected with miR-561-5p inhibitor (p<0.001).

NBR2 regulated the apoptosis, glycolysis, lactate production of MM cells, and the activation of the AMPK/mTOR pathway by targeting miR-561-5p. We inferred that NBR2 may target miR-561-5p to play a role in MM cells. Accordingly, to verify the conjecture, we implemented rescue experiments. It was observed in U266B1 cells that miR-561-5p expression was inhibited in the NBR2+MC group but was promoted in the NC+M group, while miR-561-5p expression in the NBR2+M group was higher than that in NBR2+MC group but was lower than that in the NC+M group (Figure 4A, p<0.001). Similarly, in H929 cells, miR-561-5p expression was promoted in the siNBR2+IC group but was inhibited in the siNC+I group, while the miR-561-5p expression in the siNBR2+I group was lower than that in the siNBR2+IC group but was higher than that in the siNC+I group (Figure 4B, p<0.001). In addition, based on the results of flow cytometry and the determination of both ECAR and lactate production, the co-transfection of NBR2 overexpression vector and miR-561-5p mimic reversed the effects of NBR2 overexpression and miR-561-5p mimic on the apoptosis (Figures 4C, 4E, p<0.001), ECAR (Figure 4G, p<0.001), and lactate production of MM cell line U266B1

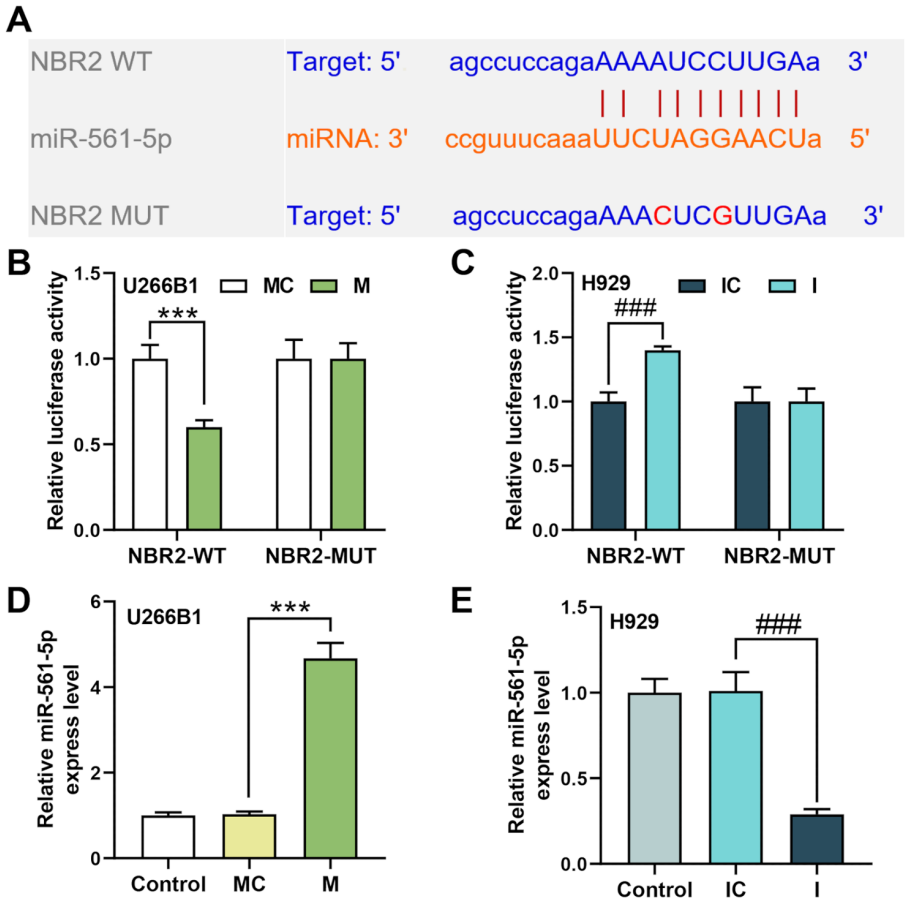

Figure 3. miR-561-5p was the target gene of NBR2. A) starBase (http://starbase.sysu.edu.cn/) predicted the binding sites between miR-561-5p and NBR2. B–C) The luciferase activity in MM cells co-transfected with NBR2 WT/MUT and miR-561-5p M/MC or I/IC was determined by dual-luciferase reporter assay. D, E) The expression level of miR-561-5p in MM cells transfected with miR-561-5p M/MC or I/IC was tested by qRT-PCR. U6 was used as an internal control. The quantified values were presented as mean ± standard deviation of at least three independent experiments. ***p<0.001 vs. MC group, ###p<0.001 vs. IC group. Abbreviations: MM-multiple myeloma; WT-wild type; MUT-mutant; M-mimic; MC-mimic control; I-inhibitor; IC-inhibitor control; qRT-PCR-quantitative real time polymerase chain reaction

(Figure 4I, p<0.01), respectively, while co-transfection of siNBR2 and miR-561-5p inhibitor reversed the respective effects of siNBR2 and miR-561-5p inhibitor on the apoptosis (Figures 4D, 4F, p<0.001), ECAR (Figure 4H, p<0.05), and lactate production of MM cell line H929 (Figure 4J, p<0.01).

The AMPK pathway has been underlined to regulate glycolysis and fatty acidification [21, 22]. Therefore, we tested the effect of the NBR2/miR-561-5p axis on the AMPK/mTOR pathway. As shown in Figures 5A–5F, NBR2 overexpression or miR-561-5p inhibitor could promote the phosphorylation of AMPK yet inhibit that of mTOR, respectively, whilst siNBR2 or miR-561-5p mimic inhibited the phosphorylation of AMPK and facilitated that of mTOR, respectively (p<0.01). However, the above-mentioned effects were reversed following the co-transfection of the NBR2 overexpression vector and miR-561-5p mimic or siNBR2 and miR-561-5p inhibitor (Figures 5A–5F, p<0.001).

The regulation of the NBR2/miR-561-5p/DLC1 axis existed in MM cells. We used Targetscan to predict the target genes of miR-561-5p (total number = 4,202), and employed starBase to predict the target genes of miR-561-5p as well (total number = 652). Compared with the normal control, there are 2,028 genes in the GEO database GSE125364 data set that were significantly lower-expressed. 66 common genes were obtained from the intersection of the three datasets (Figure 6A). Subsequently, we found that RBL1 and DLC1 in these 66 genes have regulatory effects on a variety of cancers [23, 24], including MM. Nevertheless, whether the role in MM is related to miR-561-5p remained unclear. Figures 6B and 6C showed the possible binding sites of RBL1/DLC1 and miR-561-5p. However, the miR-561-5p mimic could not inhibit the luciferase activity of cells in the RBL1-WT group but could inhibit that in the DLC1-WT group, indicating that the target gene of miR-561-5p was DLC1 rather than

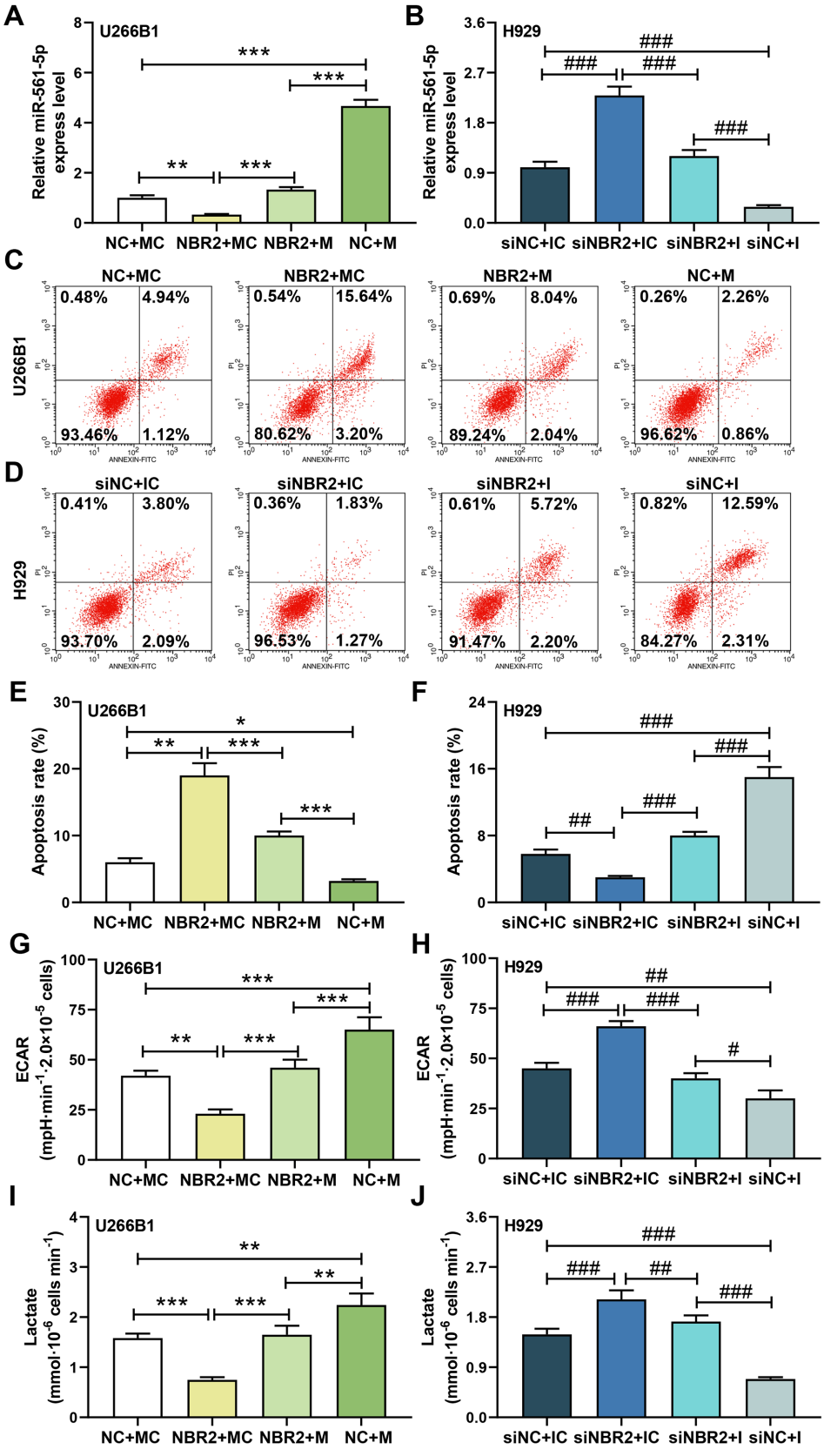

Figure 4. NBR2 regulated apoptosis, ECAR, and lactate production of MM cells by targeting miR-561-5p. NBR2 overexpression vector/NC and miR-561-5p M/MC were co-transfected into U266B1 cells, while siNBR2/siNC and miR-561-5p I/IC were co-transfected into H929 cells. A, B) The expression level of miR-561-5p in transfected U266B1 cells or H929 cells was determined by qRT-PCR. U6 was used as an internal control. C–F) The apoptosis rate of transfected U266B1 cells or H929 cells was measured via flow cytometry. G, H) The ECAR in transfected U266B1 cells or H929 cells was evaluated using the Seahorse-XF24 analyzer. I, J) The production of lactate in transfected U266B1 cells or H929 cells was calculated using the lactate assay kit. The quantified values were presented as mean ± standard deviation of at least three independent experiments.*p<0.05, **p<0.01, ***p<0.001; #p<0.05, ##p<0.01, ###p<0.001. Abbreviations: MM-multiple myeloma; ECAR-extracellular acidification rate; NC-negative control; M-mimic; MC-mimic control; I-inhibitor; IC-inhibitor control; qRT-PCR-quantitative real time polymerase chain reaction; siNBR2-NBR2 specific small interfering RNA

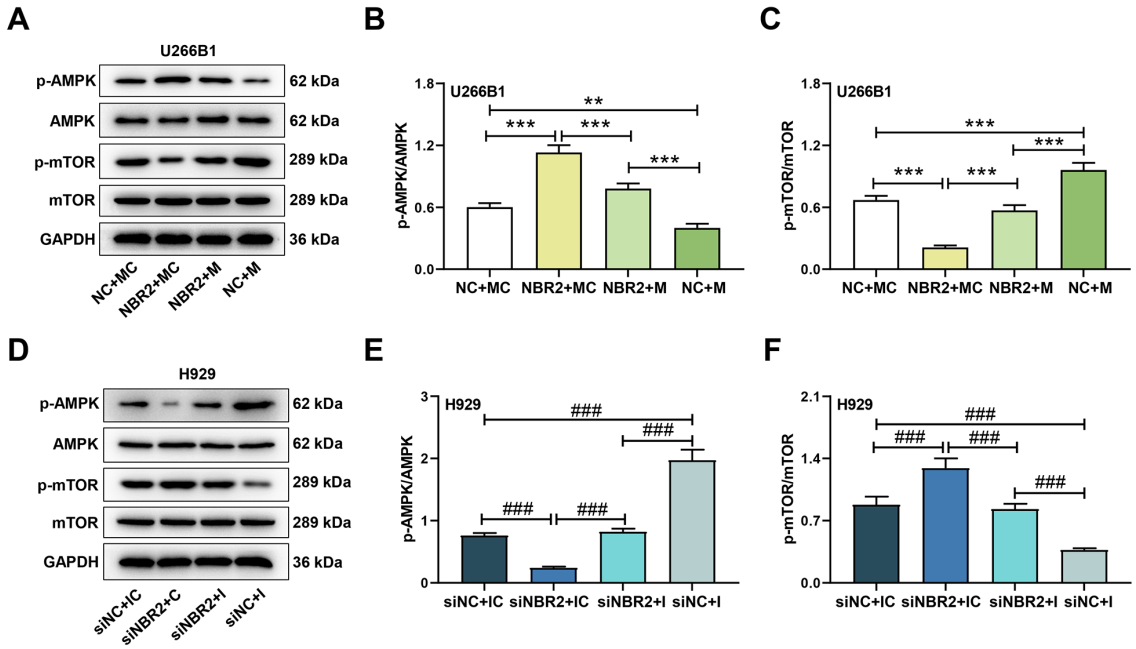

Figure 5. NBR2 regulated AMPK/mTOR signaling pathway by targeting miR-561-5p. NBR2 overexpression vector/NC and miR-561-5p M/MC were co-transfected into U266B1 cells, while siNBR2/siNC and miR-561-5p I/IC were co-transfected into H929 cells. A–F) The protein levels of p-AMPK, AMPK, p-mTOR, and mTOR in cells of these eight groups were calculated by western blot. GAPDH was used as an internal control. The quantified values were expressed as mean ± standard deviation of at least three independent experiments. **p<0.01, ***p<0.001, ###p<0.001. Abbreviations: M-mimic; MC-mimic control; I-inhibitor; IC-inhibitor control; P-AMPK-phospho-AMPK; GAPDH-glyceraldehyde-3-phosphate dehydrogenase

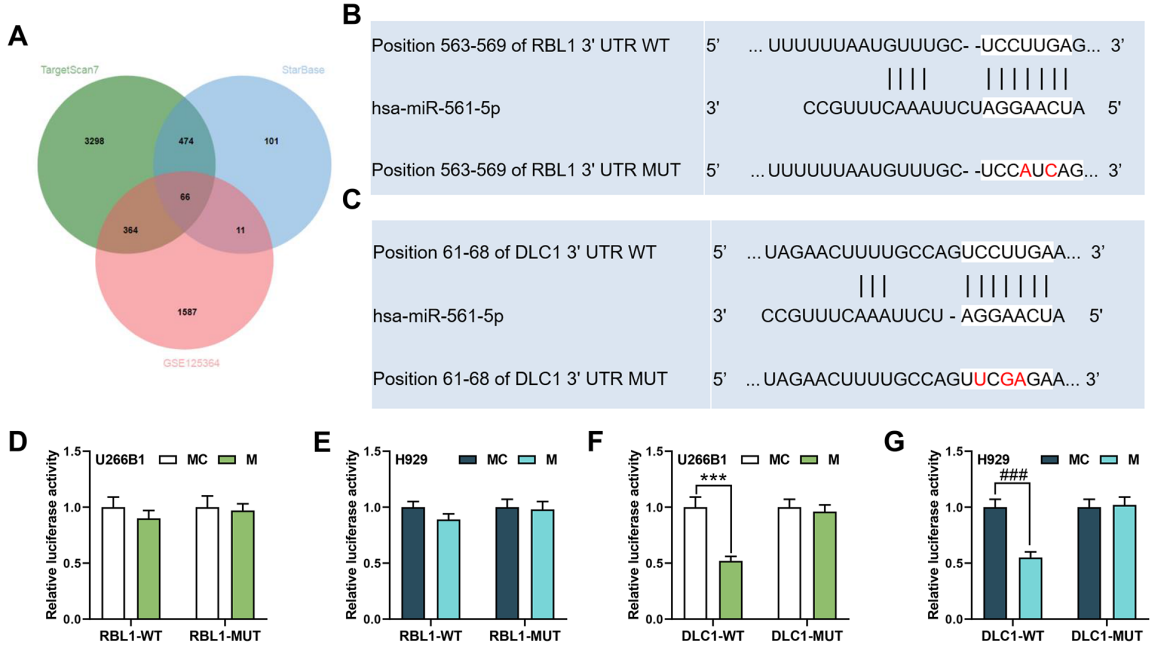

Figure 6. DLC1 was the target gene of miR-561-5p. A) Targetscan (http://www.targetscan.org/vert_72/) and starBase (http://starbase.sysu.edu.cn/) were used to predict the target genes of miR-561-5p. The mRNA that was lower-expressed compared to the normal control was analyzed through the GSE125364 data in the GEO database. A Venn diagram showed that 66 common genes were obtained from the intersection of the three. B, C) The binding site of miR-561-5p and RBL1/DLC1 was predicted by Targetscan. D–G) The luciferase activity in MM cells co-transfected with RBL1 or DLC1 WT/MUT and miR-561-5p M or MC was determined by a dual-luciferase reporter assay. The quantified values were expressed as mean±standard deviation of at least three independent experiments. ***p<0.001 vs. MC group in U266B1 cells, ###p<0.001 vs. MC group in H929 cells. Abbreviations: DLC1-deleted in liver cancer 1; MM-multiple myeloma; WT-wild type; MUT-mutant; M-mimic; MC-mimic control; qRT-PCR-quantitative real time polymerase chain reaction; GAPDH-glyceraldehyde-3-phosphate dehydrogenase

RBL1 (Figures 6D–6G, p<0.001). The experiments of western blot indicated that the protein expression level of DLC1 was increased in the NBR2+MC and siNC+I groups but was decreased in the NC+M and siNBR2+IC groups (Figures 7A–7D, p<0.05). However, miR-561-5p mimic reversed the promotive effect of NBR2 overexpression on the DLC1 expression, and miR-561-5p inhibitor reversed the inhibitory effect of silenced NBR2 on the DLC1 expression (p<0.001).

Discussion

The concept of glycolysis was first proposed by Warburg who found that the glucose metabolized by tumor tissue was more than ten times that of normal tissue [25]. At present, it is believed that the reasons for the active glycolysis of tumor cells are as follows: glycolysis can produce a lot of energy, pyruvate, and other substances, all of which are the key to the synthesis of fatty acids and nucleotides, thereby promoting cell proliferation [26]. Glycolysis will be accompanied by the production of a large amount of lactate, and the overflow of lactate outside the cell leads to the acidification of the microenvironment and enhances the resistance of tumor cells to drugs [27]. The key enzyme of glycolysis, hexokinase, can catalyze the conversion of glucose and make tumor cells more prone to glycolysis [28]. Liu et al. found that the lncRNA MALAT1/miR-1271-5p/SOX13 axis could promote the glycolysis of MM [29]. Wu et al., however, proposed that miR-489 could inhibit the aerobic glycolysis of MM and thus repress the development of MM [30]. These discoveries show that glycolysis in MM can be regulated by lncRNA or miRNA. In our research, we came up with the idea that the NBR2/miR-561-5p/DLC1 axis could regulate the production of ECAR and lactate, indicating that the development of MM can be inhibited by regulating glycolysis.

We also discovered that the mechanism by which the NBR2/miR-561-5p/DLC1 axis is involved in regulating the progression of MM is related to the activation of the AMPK/mTOR pathway to regulate glycolysis. The main sensor of cell energy status in eukaryotic cells is AMPK. A study has indicated that AMPK can affect the energy metabolism in the tumor by inhibiting the Warburg effect of tumor cells, thereby inhibiting tumor development [31]. It's believed in previous studies that activating AMPK and inhibiting mTOR can exert anti-tumor effects [32]. It has been shown that NBR2 can activate the AMPK pathway in thyroid cancer and colorectal cancer [13, 33]. Liu et al. suggested that activated AMPK could upregulate the expression of NBR2, and NBR2 could interact with AMPK to promote the activation of AMPK, thus forming a cycle, while the lack of NBR2 caused the inactivation of AMPK, thereby activating mTOR and leading to tumor development [34]. However, some recent studies have indicated that activating a low level of AMPK, under the conditions of glucose deprivation and hypoxia, can give tumor cells the advantage of survival under metabolic stress [35, 36]. Zhu et al. argued that the NBR2/miR-22/TCF7 axis can promote the malignancy of hepatoblastoma under the condition of glucose starvation [37]. This indicates

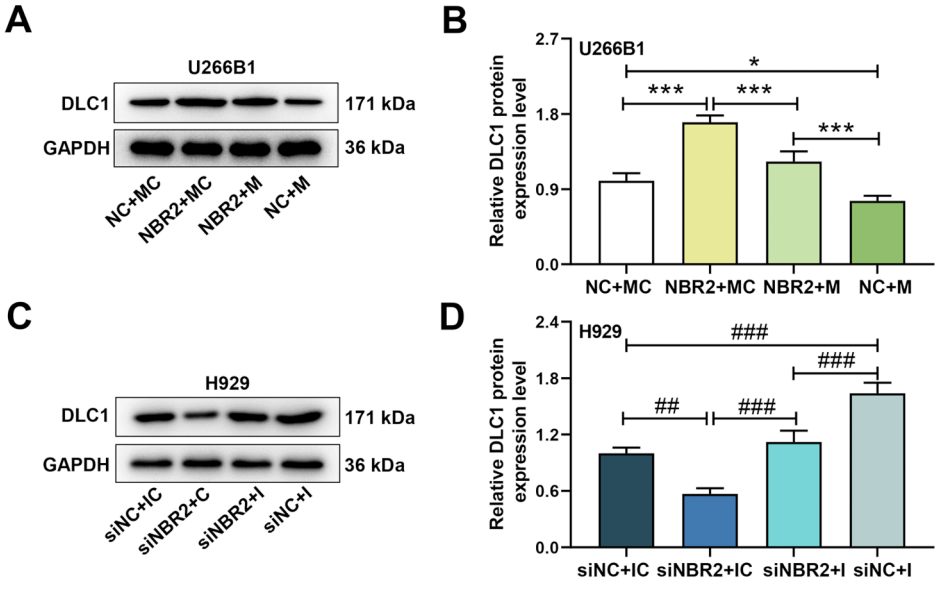

Figure 7. The expression of DLC1 was regulated by NBR2 and miR-561-5p. A–D) Western blot showed the expression of DLC1 in MM cells co-transfected with NBR2 overexpression vector and miR-561-5p M or siNBR2 and miR-561-5p I. GAPDH was used as an internal control. The quantified values were expressed as mean ± standard deviation of at least three independent experiments. *p<0.05, ***p<0.001, ##p<0.01, ###p<0.001. Abbreviations: MM-multiple myeloma, DLC1-deleted in liver cancer 1; GAPDH-glyceraldehyde-3-phosphate dehydrogenase; M-mimic; MC-mimic control; I-inhibitor; IC-inhibitor control

that the cancer-promoting or anti-tumor effect of NBR2 may depend on different metabolic states.

Ronchetti et al. proposed that miR-561 was overexpressed in MM [38]. Nevertheless, the current research on the detailed role of miR-561-5p in cancer is very rare. Chen et al. indicated that miR-561-5p was highly expressed in hepatocellular carcinoma and could promote the metastasis of cancer cells by regulating CX3CL1/CX3CR1+NK cells [39]. Our study, here, also confirmed that miR-561-5p is also higher-expressed in MM and exerts a cancer-promoting effect.

DLC1 is a tumor-suppressor gene, which was first found to be deleted in liver cancer, and in many cancers and tumor cell lines later, breast cancer and prostate cancer, for instance [40]. Ullmannova-Benson showed that the level of DLC1 was downregulated in MM, and believed that the downregulation of DLC1 level could activate Rho GTPases to cause the rearrangement of actin, thereby promoting the migration and invasion of MM [41]. We, in our study, proved that the level of DLC1 was also downregulated in MM and that increasing the expression of DLC1 through NBR2/miR-561-5p could inhibit the development of MM. A similar result has also been confirmed in other studies where Goodison et al. put forward that the migration and invasion of breast cancer cells could be inhibited by restoring the expression of DLC1 [42].

Our research, nevertheless, has some shortcomings as well. Only *in vitro* experiments were conducted in our study while *in vivo* experiments weren't conducted for further verification and further analysis of the role of NBR2 in MM is needed through bioinformatics. In addition, we found that NBR2 can inhibit MM glycolysis, but it is unclear whether the inhibitory effects of NBR2 on glycolysis are AMPK-dependent. Additionally, it remains controversial whether AMPK is an oncogene or a tumor suppressor gene. In the future, it may be necessary to knock out AMPK to further observe the effects of NBR2 on MM.

In general, our research clarified the molecular mechanism underlying the regulation of the NBR2/miR-561-5p/DLC1 axis on the growth of MM cells, which provided a theoretical basis for genetic therapy in MM.

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