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MicroRNA-769-3p Down-regulates *NDRG1* and Enhances Apoptosis in MCF-7 Cells During Reoxygenation

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Hypoxia and reoxygenation are common characteristics of solid tumors, which lead to oxidative stress and activation of stress-response genes. Previously, we observed that N-myc downstream-regulated gene 1 (*NDRG1*) was strongly down-regulated after shifting to reoxygenation, but the regulatory mechanism of *NDRG1* remained elusive. Here we focused on the regulation of *NDRG1* by microRNAs (miRNAs). Breast cancer MCF-7 cells were cultured under hypoxia for 24 h followed by 24 h of reoxygenation. The miRNA profiles were examined by Nanostring nCounter assays. Forty-three miRNAs had significant changes upon reoxygenation. *In silico* analysis identified four oxygen-sensitive miRNAs whose seed regions perfectly matched the 3′-UTR of *NDRG1*. In particular, miR-769-3p was able to inhibit the expression of *NDRG1*, which caused a significant reduction of NDRG1 protein upon reoxygenation. Furthermore, overexpression of miR-769-3p significantly inhibited cell proliferation and enhanced apoptosis. Our results revealed that miR-769-3p can functionally regulate *NDRG1* during changes in oxygen concentration.

Tumor populations need to overcome distinct microenvironmental barriers prior to metastasizing to other organs. It has been suggested that the tumor microenvironment is a unique setting for tumor progression, which requires genetic and adaptive changes in cancer cells for further survival and proliferation. Invasive cancers, therefore, could be viewed as a sequence of phenotypic adaptations to their microenvironment. The tumor microenvironment is characterized by nutrient deprivation, low pH, and hypoxia[1]. These aberrations were linked to perfusion deficits in solid tumors, which came from rapid tumor growth and profoundly disorganized vasculature[2].

Although regions of hypoxia are a common feature in solid tumors, the oxygen concentrations within a hypoxic region are highly variable. Because tumor vasculature is highly inefficient and unstable, masses of red blood cells irregularly flow to the hypoxic regions, resulting in reperfusion or reoxygenation[3]. Reoxygenation not only increases oxygen supply but also results in complex changes in cellular energy charge, oxidant generating systems, and antioxidant defenses[4]. These changes lead to oxidative stress[5], tissue damage, and activation of stress-response genes[6]. These cellular responses are activated by several key signaling pathways, such as hypoxia-inducible factor (HIF-1), nuclear factor-κB (NF-κB), activator protein 1 (AP-1), and some mitogen-activated protein kinase (MAPK) pathways[6].

Previously, we used genome-wide expression microarrays to investigate the dynamics of gene profiling during reoxygenation in breast cancer MCF-7 cells, and demonstrated that N-myc downstream-regulated gene 1 (*NDRG1*) was involved in tumor adaptation to reoxygenation[7]. *NDRG1* is a 43-kD protein composed of 394 amino acids, and is highly conserved among multicellular organisms. It is expressed ubiquitously in tissues stimulated under a wide variety of stress and cell growth-regulatory conditions, such as DNA damage[8], cellular differentiation[9], proliferation and growth arrest[10], or hypoxia[11,12]. *NDRG1* was reported to be strongly up-regulated under hypoxic conditions or nickel (Ni^{2+}) treatment via hypoxia inducible factor 1 (HIF-1), Egr-1, or Sp1[13–15]. Yet, the regulatory mechanism of *NDRG1* upon oxygen fluctuation is still unclear.

An oncogenic and tumor-promoting role of *NDRG1* has been suggested, because it is overexpressed in various human cancers, including lung, brain, skin, kidney, and breast cancers[13,16]. Since hypoxia is prevalent in many solid tumors, its regulation is governed by hypoxia-inducible factor 1 alpha (HIF-1α) and p53-dependent pathways[17,18]. *NDRG1* can be induced to promote cell migration under intermittent hypoxia[19]. However, *NDRG1* functions as a metastatic suppressor in prostate and colon cancers[9,20]. The contradictory roles of *NDRG1* in different cancers remain to be clarified, although it might be explained by their multiple cellular localizations and complex regulation by diverse physiological and pathological factors.

MicroRNAs (miRNAs), 18–25 nucleotides in length, are a class of non-protein-encoding small RNAs[21]. MiRNAs originate from longer primary miRNA transcripts located in either intergenic or intronic regions[22]. Intronic miRNAs are co-expressed with the genes in which they are located and are regulated from the same promoters as their host genes[22]. It has been indicated that miRNAs regulate the expression of more than 30% of protein-coding genes[23], and that miRNAs control multiple biological and metabolic processes, ranging from tissue development and signal transduction to the disease processes of tumorigenesis and cancer progression[24,25]. Regulation of gene expression by miRNAs is achieved by translation repression, direct mRNA degradation or miRNA-mediated mRNA decay[26]. In a majority of cases, miRNAs bind with imperfect complementarity, with the exception of their 'seed region', to their targeted mRNAs at the 3′-UTR by the RNA induced silencing complex (RISC). MiRNA-RISC complex may interact with various translation factors, such as eIF4F, and inhibit the initiation and/or elongation of protein translation[27]. MiRNAs can also directly degrade their targeted mRNAs or destabilize mRNAs through de-adenylation and/or de-capping of targeted mRNAs[28].

Although >2,000 miRNAs have been discovered in mammals, the functions of most miRNAs are not understood. Recent studies revealed that a significant proportion of hypoxia-regulated miRNAs were overexpressed in human cancers and could affect important processes, such as apoptosis, proliferation, and angiogenesis[29]. For instance, miR-210, miR-199a, and miR-34a-5p were reported to regulate hypoxia and angiogenesis[30–32]. MiR-182 promoted proliferation and invasion of human prostate cancer cells by directly suppressing *NDRG1*[33].

Since the regulatory mechanism of *NDRG1* upon reoxygenation remains elusive, we investigated whether the down-regulation of *NDRG1* upon reoxygenation was achieved partly through miRNA regulation. Thus, miRNA expression upon reoxygenation was examined using NanoString nCounter® miRNA Expression Assays and analyzed by *in silico* analysis. We discovered that, upon reoxygenation, miR-769-3p can directly inhibit *NDRG1*, resulting in reduced proliferation and enhanced apoptosis in MCF-7 breast cancer cells.

Results

Previous studies have reported that the expression levels of miRNAs were changed under hypoxia[34,35]. To investigate whether *NDRG1* is regulated by miRNA under different O_2 conditions, we first used a hybridization-assisted, expressed sequence tag (EST)-based technique, the NanoString nCounter™ Analysis System, to identify differentially expressed miRNAs during reoxygenation. MCF-7 cells were grown in hypoxia for 24 h, and harvested at 0, 1, 12, and 24 h after reoxygenation to measure the expression profiles of miRNAs. Fold change assessments (>1.5×) and t-tests ($P < 0.05$) of each time point as compared to the hypoxia control (time 0) were applied for selecting oxygen-responsive miRNAs. We identified 43 miRNAs passing these criteria, with 63% (n = 27) of the miRNAs up-regulated upon reoxygenation. The heat map of these differentially expressed miRNAs is shown in Fig. 1A. Because miRNAs usually negatively regulate their target genes, and because *NDRG1* was down-regulated upon reoxygenation, the up-regulated miRNAs were chosen for further investigation.

In order to examine which miRNAs directly regulate *NDRG1*, a bioinformatics algorithm, Target Scan (http://www.targetscan.org), was used to search the binding sites of miRNAs in the *NDRG1* 3′-UTR. Among the 27 up-regulated miRNAs, the seed regions of miR-769-3p, miR-501-3p, miR-2276, and miR-1282 matched perfectly the *NDRG1* 3′-UTR sequence. Therefore, we validated the expression of these four miRNAs by quantitative RT-PCR. As shown in Fig. 1B–E, the expression levels of miR-769-3p, miR-501-3p, miR-2276, and miR-1282 were all significantly up-regulated at 24 h under reoxygenation, showing that these miRNAs are responsive to reoxygenation.

Furthermore, molecular dynamics simulation and thermodynamics analysis were used to predict the strength of binding affinity[36]. The results showed that miR-769-3p had the highest alignment score and the most favorable thermodynamics (i.e., lowest G score) (Table 1). Therefore, we focused on miR-769-3p for the following experiments.

First of all, we examined the endogenous expression levels of miR-769-3p and its precursor in breast cancer MCF-7 cells at different oxygen concentrations. As shown in Fig. 2A, miR-769-3p was up-regulated during normoxia and reoxygenation as compared to hypoxia, indicating that miR-769-3p is an oxygen-dependent miRNA. However, the expression levels of precursor mir-769-3p in hypoxia were not significantly different from those in normoxia and reoxygenation (Fig. 2B).

Next, in order to explore whether miR-769-3p could directly bind and regulate *NDRG1*, we used computational algorithms[37] to predict the potential binding sites in the *NDRG1* 3′-UTR and examined their interaction using luciferase assays. The location of the potential binding site was 1,014–1,036 bp downstream relative to the start site of *NDRG1* 3′-UTR (Fig. 3A). Because the seed region of the miRNA, which includes 2 to 8 nucleotides at the 5′-end of the miRNA, must be complementary to the 3′-UTR of target genes, we mutated five nucleotides in the binding site to validate their interaction (Fig. 3A). By co-transfecting the miR-769-3p plasmids with the reporter construct, which contained the *NDRG1* 3′-UTR behind the luciferase gene (Fig. 3A), to HEK 293 cells, we observed that, in normoxia, miR-769-3p was able to inhibit the luciferase activity as compared to the miR-empty vector control (Fig. 3B). When the binding site was mutated, the luciferase activity was recovered (Fig. 3B). These results suggest that *NDRG1* is the target of miR-769-3p.

Afterwards, we overexpressed miR-769-3p in MCF-7 cells (Fig. S1) and examined the mRNA and protein expression of NDRG1 upon reoxygenation. As expected, overexpression of miR-769-3p led to a significant reduction of both *NDRG1* mRNA (Fig. 4A) and protein (Fig. 4B) upon reoxygenation. These data indicate that down-regulation of NDRG1 is at least partly regulated by miR-769-3p under reoxygenation.

Lastly, previous studies report that silencing *NDRG1* can reduce proliferation. Therefore, we hypothesized that overexpression of miR-769-3p could also reduce proliferation through inhibiting *NDRG1*. As shown in Fig. 5A, cell proliferation was indeed suppressed under normoxia, hypoxia, and reoxygenation in the presence of ectopic miR-769-3p using MTT assays. Furthermore, flow cytometry analysis showed that miR-769-3p-expressing cells exhibited more late apoptosis at 24 h of normoxia (O_2), hypoxia (N_2), and reoxygenation (Re-O_2) than control cells using Annexin V binding assays (Fig. 5B & 5C). In addition, at 24 h of reoxygenation, there were higher proportions of miR-769-3p-expressing cells in sub-G1 phase than control cells (Fig. 5D & 5E). These results suggest that miR-769-3p can reduce proliferation and enhance apoptosis by inhibiting *NDRG1* upon reoxygenation.

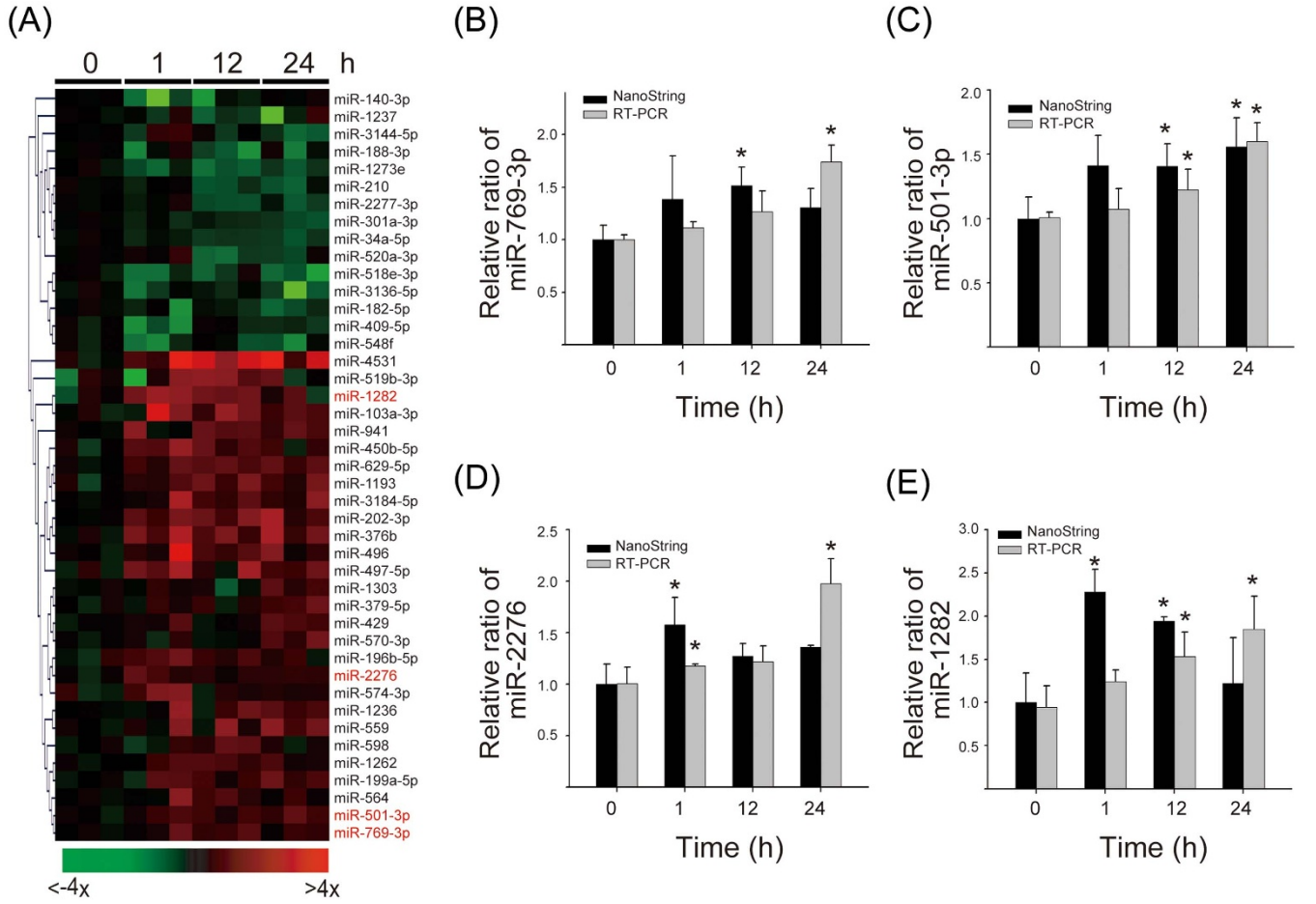

Figure 1 | Identification of miRNAs that were differentially expressed during reoxygenation. (A) Heatmap of the differentially expressed miRNAs during reoxygenation (re-O_2) using NanoString nCounter® miRNA Expression Assays. Red: up-regulated in re-O_2 as compared to hypoxia control (0 h); green: down-regulated in re-O_2. (B–E) NanoString nCounter® miRNA Expression Assays and real-time PCR validation of miR-769-3p (B), miR-501-3p (C), miR-2276 (D), and miR-1282 (E) in re-O_2. *RNU44* was used as an internal control for real-time PCR. Data in the bar chart are the mean ± SD for three independent experiments.*, $P < 0.05$.

Discussion

In this study, we used a genome-wide approach to screen all miRNAs and identified 43 oxygen-sensitive miRNAs in MCF-7 breast cancer cells. Among these oxygen-sensitive miRNAs, we demonstrated that miR-769-3p could directly inhibit *NDRG1*. Also, miR-769-3p could enhance apoptosis and suppress cell proliferation upon reoxygenation.

Previous studies have used miRNA microarrays to screen all miRNAs and identify hypoxia-related miRNA candidates[34,38]. However, in our pilot study, many previously reported hypoxia-related miRNAs could not be validated to regulate *NDRG1*. There are at least two possible reasons for this. First, since the previous microarrays used hybridization between fluorescent miRNAs and probes, and the relative expression levels were quantified by intensity of fluorescence, the microarrays had low sensitivity and limited dynamic range. Therefore, the fluorescence signals were amplified using PCR/in vitro transcription, which may have generated non-linear amplification and led to misrepresentation of the quantitative transcript values[39,40]. A second possible reason why previous microarray approaches failed to identify more *NDRG1*-regulatory miRNAs is a lack of probes to detect newly discovered miRNAs. Therefore, we used a new version of the NanoString nCounter® Analysis System, a sequence-tag counting-based technology, to do a genome-wide screen in this study. The advantages of NanoString are that it has higher sensitivity and dynamic range, and does not require amplification or labeling of miRNA[41].

Through the screening of oxygen-responsive miRNAs, 43 miRNAs had dramatic changes as compared to 0 h upon reoxygenation. Among these 43 miRNAs, miR-210 and miR-34a-5p were known to be up-regulated in hypoxia signaling pathways[30,42]. Hence, miR-210 and miR-34a-5p should be down-regulated upon reoxygenation, which was consistent with our Nanostring results (Fig. 1A).

Based on the results of sequence analysis, molecular dynamics simulation and thermodynamics analysis, miR-769-3p was found to be the most likely miRNA to regulate *NDRG1* and hence was chosen for further validation. MiR-769-3p was first discovered in 2006[43]. The location of miR-769-3p is in the first intron of *CCDC61*. However, the role of miR-769-3p has not previously been

Table 1 | The molecular dynamics simulations and thermodynamics analysis

miRNA	Alignment score	*P*-value†	G score#
hsa-miR-769-3p	106.5*	1.7×10^{-3}	−13.64
hsa-miR-1282	92.5*	8.2×10^{-3}	−9.81
hsa-miR-501-3p	89.5	5.7×10^{-2}	−9.10
hsa-miR-2276	89.5	1.0×10^{-1}	−9.98

†Probability of alignment score was empirical probability from 10,000 permutations.
#G scores are thermodynamic free energy scores.
*Significant ($P < 0.05$).

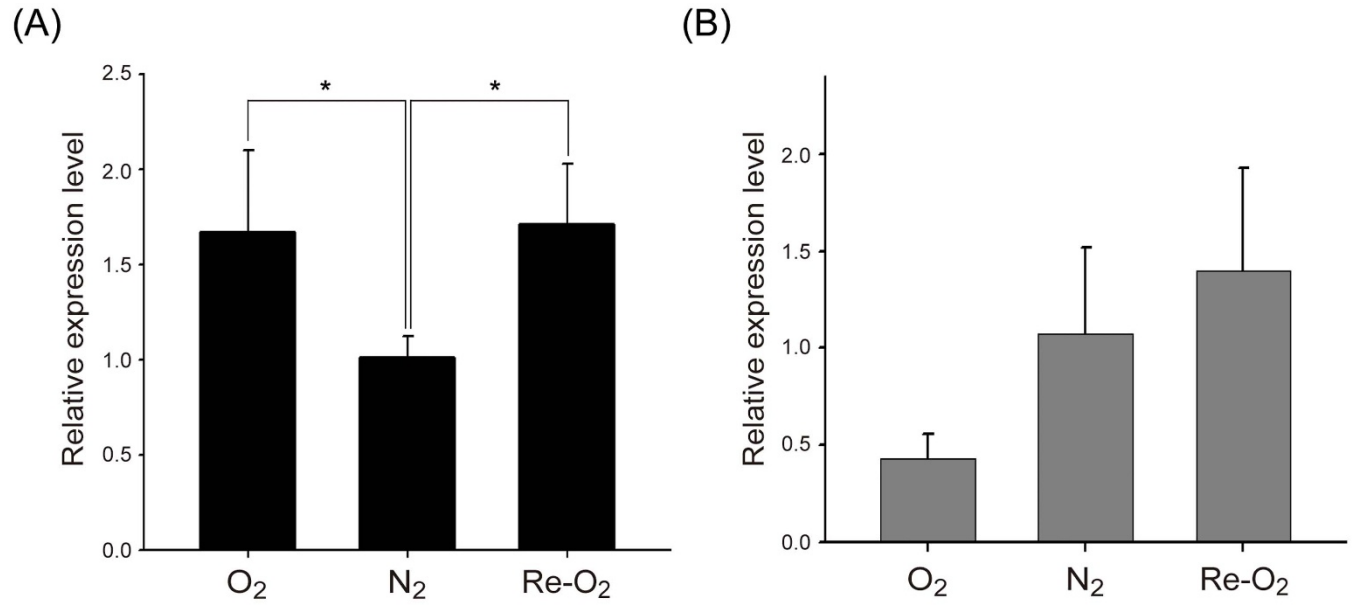

Figure 2 | MiR-769-3p is down-regulated in hypoxia and up-regulated upon re-oxygenation. (A) Relative expression levels of miR-769-3p in normoxia (O_2), hypoxia (N_2), and reoxygenation (Re-O_2) using quantitative RT-PCR. Breast cancer MCF-7 cells were cultured under normoxia, hypoxia for 24 h, or 24 h of hypoxia followed by 24 h of reoxygenation. The relative expression level of each condition was compared to hypoxia; 18s rRNA was used as an internal control. *: $P < 0.05$. (B) Relative expression levels of precursor mir-769-3p at different oxygen levels using quantitative RT-PCR.

reported. In this study, we demonstrated that miR-769-3p could down-regulate *NDRG1* by degrading the mRNA of *NDRG1* and inhibiting its protein levels when oxygen was available. This also implied that miR-769-3p was dissociated from *NDRG1* under hypoxia. Yet, the detailed mechanism of the oxygen-sensitive association of miR-769-3p with *NDRG1* needs further exploration. Furthermore, miR-769-3p not only regulated *NDRG1* but was also involved in other hypoxia/reoxygenation-responsive pathways. According to the computational prediction of the function of miR-769-3p's target genes[37], miR-769-3p may be involved in c-Myc's transcription factor network, the Rac-1signaling pathway, the MAPK pathway, and the HIF-2α transcription factor network, via targeting to *PGK1*, *MAPK14*, *CDKN1A* (P21), and *SP1*, which are upstream or downstream of *NDRG1* in these signaling pathways.

Overexpression of miR-769-3p led to a significant reduction of NDRG1 protein and mRNA upon reoxygenation, but not in hypoxia (Fig. 4B). This result suggested that miR-769-3p alone was not sufficient to reduce NDRG1 levels in hypoxia. We can envision two possible scenarios. First, up-regulation of *NDRG1* under hypoxia may not be regulated solely by miRNAs, in which case the enormous expression of *NDRG1* in hypoxia by other transcriptional regulations may offset the inhibitory effect of ectopic miR-769-3p on *NDRG1*. Second, since miR-769-3p may have many target genes in addition to *NDRG1*, the miR-769-3p-regulated genes may compete with *NDRG1*

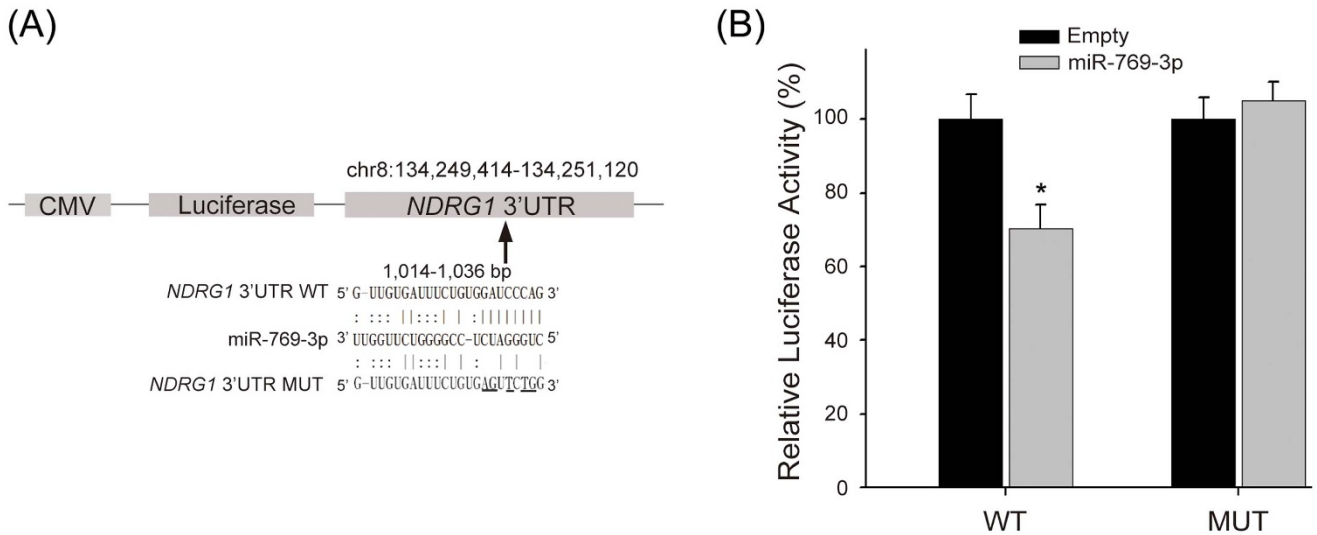

Figure 3 | MiR-769-3p directly regulates *NDRG1* activity. (A) Schematic representation of the *NDRG1* 3′-UTR expression plasmid and the miR-769-3p target sequence. Firefly luciferase constructs contained the CMV promoter, luciferase coding region, and a fragment of the *NDRG1* 3′-UTR wild-type (WT) or mutant (MUT). The mutation sites were underlined. (B) Luciferase assays of miR-769-3p co-transfected with firefly luciferase constructs containing *NDRG1* 3′-UTR wild-type or mutant into HEK 293 cells. *Renilla* luciferase was used as the transfection control. The relative luciferase activity represented the dual luciferase activity ratio (firefly/*Renilla* luciferase). *: $P < 0.05$.

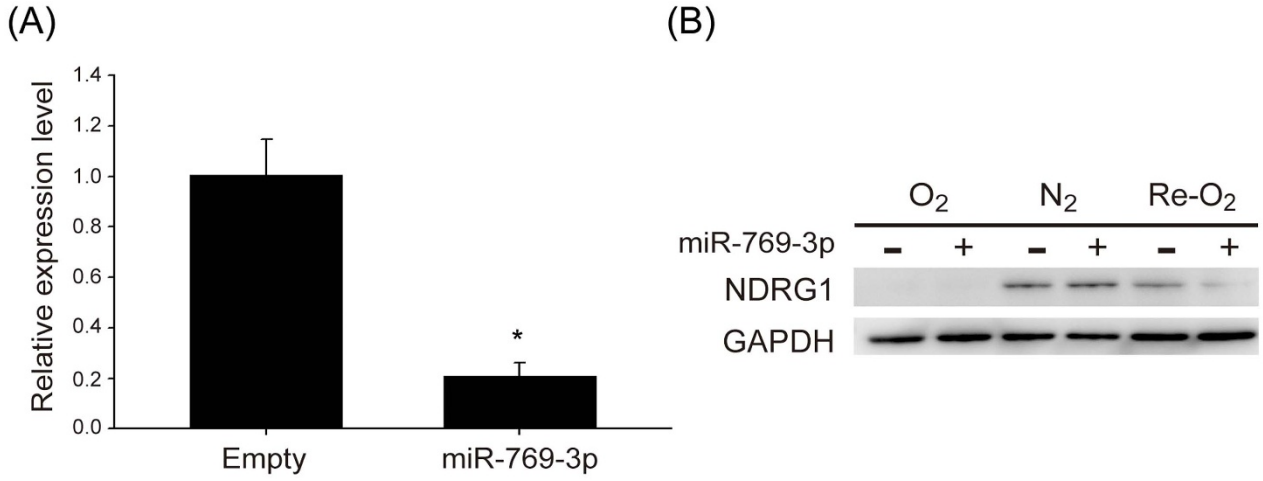

Figure 4 | Overexpression of miR-769-3p suppresses *NDRG1* expression. (A) Relative expression levels of *NDRG1* in MCF-7 cells overexpressing pri-mir-769-3p in normoxia using quantitative RT-PCR; 18S rRNA was used as a loading control. (B) Western blot analysis of NDRG1 in MCF-7 cells overexpressing pri-mir-769-3p in normoxia (O_2), hypoxia (N_2), and reoxygenation (Re-O_2). GAPDH was used as an internal control. Full-length blots/gels are presented in Supplementary Figure 2 & 3.

for miR-769-3p. These RNAs containing the same miRNA binding sites in their 3′-UTR are referred to as competing endogenous RNAs (ceRNAs), which could regulate each other by competing for the shared miRNAs[44,45]. Therefore, when ceRNAs were up-regulated, more miR-769-3p bound to these ceRNAs, which led to less inhibition of *NDRG1* and subsequently up-regulated *NDRG1*. According to computational predictions[37], miR-769-3p could target *MAPK14*, *PGK1*, and *SP1*, which all have increased expression in hypoxia. Therefore, the up-regulation of *MAPK14*, *PGK1*, and *SP1* could compete with *NDRG1* for miR-769-3p, and weaken its inhibition of *NDRG1* in hypoxia.

Regarding the functions of miR-769-3p, we showed that overexpression of miR-769-3p could inhibit cell proliferation and induce cell apoptosis (Fig. 5). However, miR-769-3p might regulate hundreds of mRNAs. Whether the induction of apoptosis and inhibition of cell proliferation were solely through the *NDRG1* signaling pathway is not currently known. Yet, we found that the patterns of proliferation and apoptosis were similar in MCF-7 cells transfected with *NDRG1* wild-type 3′-UTR and mutant 3′-UTR in normoxia, hypoxia, and reoxygenation (data not shown), implicating that *NDRG1* was not the only target gene of miR769-3p. That is, the effects of miR-769-3p on cell proliferation and apoptosis were not solely dependent on *NDRG1*. In summary, this study is the first one to report the function of miR-769-3p, and demonstrates that miR-769-3p can functionally regulate *NDRG1* upon reoxygenation.

Methods

Cell culture. Human breast adenocarcinoma cell line MCF-7 was obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (Life Technologies) and 1% antibiotic solution (penicillin-streptomycin-amphotericin solution, Biological Industries, Beit-Haemek, Israel) at 37°C in a humidified incubator under 5% CO_2. For hypoxia cultures, cells were incubated in a hypoxia chamber (InVivo$_2$-200, Ruskinn Technology, Leeds, UK) for 24 h with 0.5% O_2, 5% CO_2 and residual N_2. After 24 h of hypoxia, cells were shifted to a humidified incubator with 5% CO_2 and 95% air, and incubated at 37°C. The samples were harvested at selected time points after reoxygenation.

Nanostring data analysis. The nCounter® miRNA Expression Assays kit (Nanostring, Seattle, WA, USA) was utilized to perform miRNA expression profiling. Each miRNA was detected by ligating the unique digital tags which contain a specific digital barcode representing the counts of miRNAs. The workflow of normalization was according to the manufacturer's instructions. The samples were normalized to the geometric mean of the 100 miRNAs with the highest counts. The O_2-responsive miRNAs were selected when their expression change was significantly ($P < 0.05$) greater than 1.5-fold at a given time point as compared to the hypoxia control (0 h).

Quantitative reverse transcription PCR. Total RNA was extracted using TRIZOL Reagent (Ambion, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription of miRNA was performed using the TaqMan MicroRNA Reverse Transcription kit (Life Technologies). The resulting cDNA was detected using the FastStart Universal SYBR Green Master Mix (Roche, Branchburg, NJ, USA) or Universal ProbeLibrary Probe #21 (Roche) with a 7900HT Fast Real-Time PCR system (Life Technologies). *RNU44* was used as an endogenous control to normalize the expression of miRNA. The following primers were used to detect miRNA expression: miR-769-3p: 5′- GCGGCGGCTGGGATCTCCGGGGTC -3′ (forward), 5′- GTGCAGGGTCCGAGGT -3′ (reverse); miR-1282: 5′- GCGGCGGTCGTT-TGCCTTTTT -3′ (forward), 5′- GTGCAGGGTCCGAGGT -3′ (reverse); miR-501-3p: 5′- GCGGCGGAATGCACCCGGGCAAG -3′ (forward), 5′- GTGCAGGGTCC-GAGGT -3′ (reverse); miR-2276: 5′- GCGGCGGTCTGCAAGTGTCAGAG -3′ (forward), 5′- GTGCAGGGTCCGAGGT -3′ (reverse); *RNU44*: 5′- TCGCGCC-TGGATGATGATAGC -3′ (forward), 5′- GTGCAGGGTCCGAGGT -3′ (reverse).

Construct design and cell transfection. *MiRNA-expressing vector.* The primary sequence of miR-769-3p, including the flanking precursor sequence (150 bp long), was amplified from human leukocyte DNA. The following primers were used: miR-769-3p: 5′- CATGGATCCAGGATGCCTTTGGCTGTGT -3′ (forward) and 5′-CATAGATCTACACCTCCCCTCCTTCTCTC -3′ (reverse). The PCR product was gel-purified and cloned into the pcDNA6.2-GW/EmGFP-miR-neg vector (Life Technologies).

Luc-NDRG1 vector. The *NDRG1* 3′-UTR was amplified by PCR from genomic DNA isolated from human blood with the following primers: 5′-CATACTAGTGCGGCCTGCCCAGCTGCCGC -3′ (forward) and 5′-CATGCCGGCCCGCTGCAAAGTTACAA -3′ (reverse). The pMIR-*NDRG1*-3′-UTR construct was digested with *Spe*I and *Nae*I, and the generated fragment was inserted into the *Spe*I-*Nae*I sites of the pMIR-REPORT miRNA Expression Reporter Vector (Life Technologies). The miR-769-3p binding site (1,014–1,036 bp downstream relative to the start site of 3′-UTR of *NDRG1*) was predicted using miRSystem[37]. Five nucleotide mutations of the miR-769-3p binding site were made in the *NDRG1* 3′-UTR as a negative control with no binding. The Luc-NDRG1 vector was amplified by PCR with KAPA HiFi HotStart polymerase (KAPA Biosystems, Woburn, MA, USA) with mutated primers. After amplification, the template plasmid DNA with methylation was eliminated by *Dpn*I endonuclease (New England Biolabs, Inc., Ipswich, MA, USA). The mutated plasmids were then transformed into *E. coli* for amplification, extracted, and sequenced for validation.

Cell transfection. MCF-7 cells were seeded in antibiotic-free medium at 70–80% confluence. The cells were transfected with the miR-769-3p-expression vector using TransIT®-2020 Transfection Reagent (Mirus Bio LLC, Madison, WI, USA) according to the manufacturer's instructions.

Luciferase reporter assay. HEK293 cells were co-transfected with 300 ng of miR-769-3p, 100 ng of the reporter vector containing the *NDRG1* 3′-UTR or the mutant 3′-UTR, and 25 ng of the *Renilla* luciferase vector as a transfection control. After 48 h of transfection, the cells were collected, and the luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Western blot. The cells were washed twice with phosphate-buffered saline (PBS) (Life Technologies) and lysed in RIPA lysis buffer (Sigma, St. Louis, MO, USA). Protein

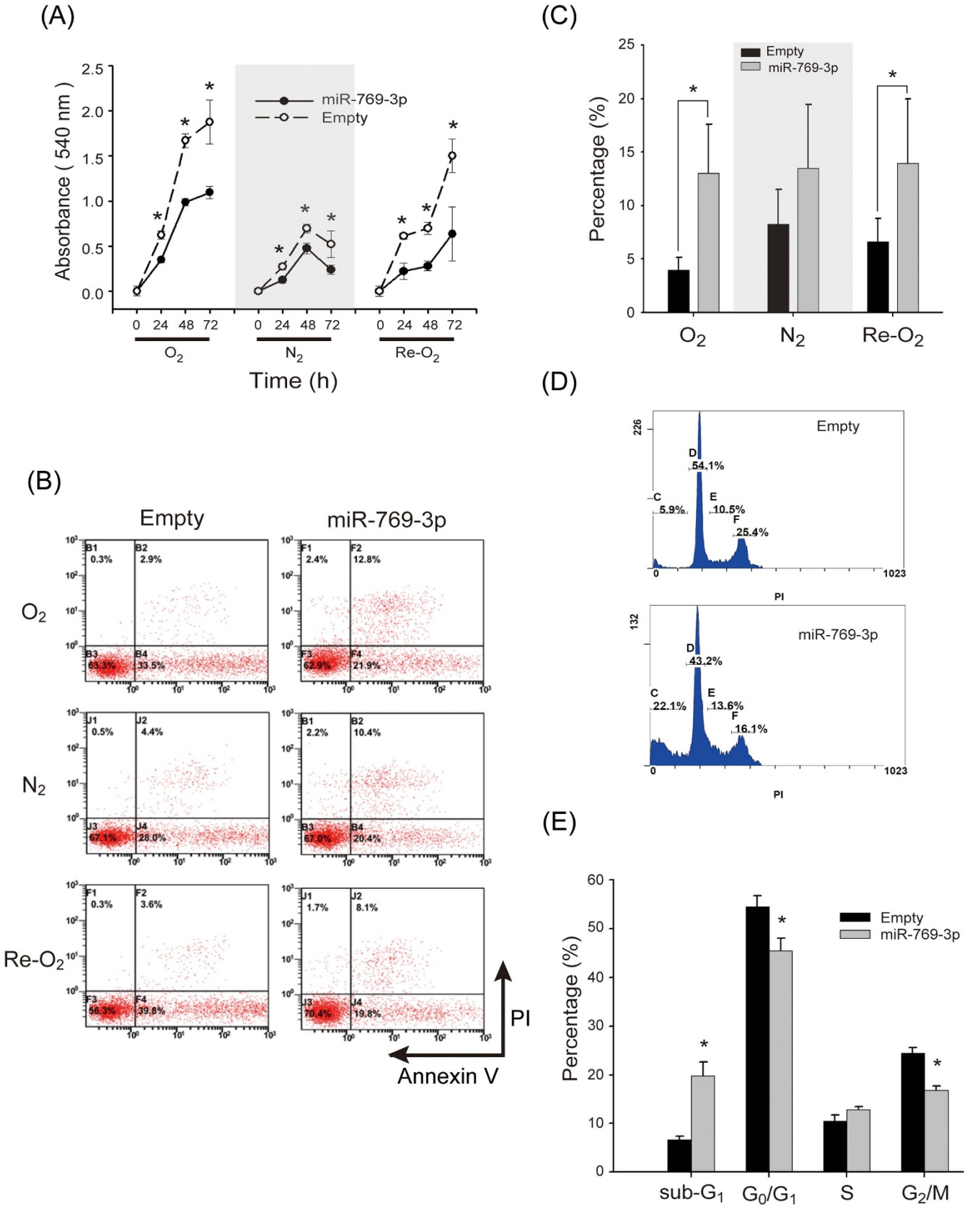

Figure 5 | Overexpression of miR-769-3p suppresses proliferation and induces apoptosis of MCF-7 breast tumor cells. (A) MTT assays of MCF-7 cells overexpressing miR-769-3p in normoxia (O_2), hypoxia (N_2), and reoxygenation (Re-O_2). (B) A representative diagram of annexin V binding assay at 24 h of O_2, N_2, and Re-O_2. Left panel: empty vector; right panel: MCF-7 cells overexpressing miR-769-3p. (C) Flow cytometry analysis for apoptosis at 24 h of O_2, N_2, and Re-O_2. using annexin V and propidium iodide in MCF-7 cells overexpressing miR-769-3p. (D) A representative diagram of flow cytometry for analyzing cell cycle at 24 h of reoxygenation. Upper panel: empty vector; lower panel: MCF-7 cells overexpressing miR-769-3p. (E) Quantitative graph of flow cytometry results in panel (D) showing the percentage of cells in each phase of the cell cycle. *, $P < 0.05$.

concentrations were determined using the Protein Assay Reagent (Bio Rad Laboratories, Hercules, CA, USA). Protein samples (30 μg) were loaded on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Bio Rad Laboratories, Inc.). Blocking was performed with 3% nonfat milk in a 1× mixture of Tris-buffered saline. The membranes were incubated overnight at 4°C with antibodies to NDRG1 (Abcam Inc., Cambridge, MA, USA) and GAPDH (GeneTex, Irvine, CA, USA). After washing and incubation with secondary antibodies for 1 h at room temperature, blotted proteins were detected using an enhanced chemiluminescence (ECL) system (Millipore, Billerica, MA, USA) with the BioSpectrum Imaging System (UVP, Upland, CA, USA).

MTT assay. Cells were seeded at a density of 3.0×10^4 cells/400 μl in a 24-well plate. After 12 h of incubation, cells were incubated with 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) solution (Sigma) (1 ml per well of 5 mg/ml solution in PBS) for 3 h. Next, 400 μl/well of DMSO (Sigma) was added to dissolve the converted purple formazan, and the absorbance of formazan was measured at 540 nm using an enzyme-linked immunosorbent assay (ELISA) reader (BioTek, Winooski, VT, USA).

Flow cytometry analysis of cell cycle and cell death. MCF-7 cells were seeded at a density of 4×10^5 cells/well in a 6-well plate and transfected with miR-769-3p plasmid. After transfection, cells were incubated in hypoxia for 24 h and shifted to normoxia for 24 h. Samples were trypsinized, resuspended in PBS (Life Technologies), and fixed with cold 70% ethanol overnight. The DNA contents were evaluated after staining with propidium iodide (PI) solution containing 50 μg/ml propidium iodide (PI) (Sigma), 0.1 mg/ml RNase A (Sigma), 0.05% Triton X-100 (Sigma) in PBS (Life Technologies). Cell cycle analysis was carried out using a Beckman Coulter FC500 (Beckman, Brea, CA, USA) and CellQuest software.

The FITC Annexin V Apoptosis Detection Kit (BD Pharmingen, San Jose, CA, USA) was used to detect apoptotic cells by flow cytometry. Annexin V binding buffer was used to resuspend cells, and the cell suspensions were stained with FITC-annexin V and PI staining solution for 15 min at room temperature. The apoptotic/necrotic cell population was analyzed with a Beckman Coulter FC500 (Beckman).

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Author contributions

L.C.L. and Y.P.S. conceived and designed the experiments. E.C.L., Y.C.C. and W.Y.H. performed the experiments. E.C.L., Y.P.S., L.L.C. and Y.C.C. analyzed the data. Y.P.S., M.H.T., E.Y.C. and L.C.L. contributed reagents, materials, and/or analysis tools. E.C.L. and L.C.L. wrote the paper. All authors reviewed the manuscript.

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