Proceedings of the 2nd International Conference on Modern Medicine and Global Health
DOI: 10.54254/2753-8818/32/20240824

Discovery and validation of putative anticancer genes in lung adenocarcinoma

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Abstract. Lung adenocarcinoma (LUAD) is a significant subtype of lung cancer, and understanding the roles of tumor suppressor genes in LUAD remains crucial. In this study, we aimed to identify and experimentally validate potential tumor suppressor genes selected from online databases. By analyzing the TCGA cancer database, we identified genes exhibiting significant differential expression between normal and tumor tissues in LUAD, particularly focusing on genes with lower expression levels in tumors associated with shortened survival periods. Subsequently, we selected *UNC5B*, *DAB2IP*, *SEMA3F*, *PPM1L*, and *AXIN2* as candidate genes and performed knockdown experiments using LUAD cell line HCC827. Our findings revealed that all selected genes, except UNC5B, conferred tumor-like properties when knocked out, suggesting their potential as anti-tumor agents. These results contribute to the understanding of LUAD pathogenesis and highlight the significance of studying tumor suppressor genes in the context of cancer development and patient survival.

Keywords: Lung adenocarcinoma, Tumorigenesis, Knock out, Gene expression

1. Introduction

Genetic factors are a critical cause of tumorigenesis, which involves multiple oncogenes including oncogene and anti-oncogene genes [1, 2]. Multiple proto-oncogenes are activated through different pathways in different spaces and times as well as different tumor suppressor genes mutations are enriched and lose the regulation of cell growth, driving the malignant progression of tumors [3]. The strategy of knocking out (KO) a gene of interest in cultured cells can be used to study gain of function and loss of function phenotypes. Lung Adenocarcinoma remains one of the deadliest cancers in the world, with non-small cell lung cancer (NSCLC) constituting approximately 80-85% of all cases of lung cancer [4, 5]. Though many genes have been implicated as major tumor suppressors in Lung Adenocarcinoma [6, 7], there still exists gaps where the roles of genes are unconfirmed, only suspected based on their high frequency of inactivation in patients and correlation with low patient survival rates when underexpressed [8]. Thus, in this study we aim to determine whether genes selected from online databases with bioinformatics methods experimentally supported to be tumor suppressors.

Based on an analysis of the TCGA cancer database, we identified a group of genes that exhibit significant differential expression between normal and tumor tissues in Lung Adenocarcinoma (LUAD). After a detailed examination of the gene expression profiles, we singled out a subset of genes with lower expression levels in tumor tissues. Subsequently, in conjunction with gene survival curve analysis, we focused on genes whose low expression is associated with shortened survival periods. These genes were

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chosen as candidates for subsequent experimental validation on the LUAD cell line HCC827 to ascertain their potential roles as tumor suppressors. Knockdown (KD) of a gene of interest in cultured cells strategies can be used to study either gain of function or loss of function phenotypes.

Thus we hypothesize that genes exhibiting differential expression between normal and tumor tissues may constitute a genetic predisposition factor contributing to the development of cancer. This hypothesis underscores the importance of comparing gene expression profiles within the same organ, specifically targeting genes with diminished expression in tumor cells. Furthermore, it is postulated that genes demonstrating lower expression levels in tumors may correlate with reduced survival rates in cancer patients, implicating their potential significance as putative tumor suppressor genes.

Based on the TCGA analysis, we selected *UNC5B, DAB2IP, SEMA3F, PPM1L*, and *AXIN 2* as candidate genes due to their correlation with Lung Adenocarcinoma dysfunction and low patient survival rates. We then constructed siRNAs targeting various anti-tumor genes with which HCC827 cells were transfected. Finally, we performed a series of validation assays to evaluate the genes' anti-tumor effectiveness, and find that, with the exception of UNC5B, all genes conferred tumor-like properties to cells when knocked out, thus indicating their anti-tumor properties.

2. Methods

2.1. Anti-tumor Candidate Genes Were Selected from Genome Database

The goal of this step is to identify cooperating anti-tumor gene at genomic scale. First, several genes are selected based on their high frequency of being under-expressed in lung adenocarcinoma and their correlation with low patient survival rates when under-expressed from online databases (https://bioinfo.uth.edu/TSGene/index.html?csrt=12581143753607606140, https://www.cbioportal.org/), and GEPIA 2 (cancer-pku.cn).

2.2. Cell Transfection

Cells were inoculated in 12-well plates and transfected with siRNA (1 nM) using lipo3000 transfection reagent for 72 hours [9]. The culture medium was then discarded, and the cells were fixed by adding 11% glutaraldehyde and shaken on an oscillator for 20 minutes. The fixed cells were washed three times with deionized water and then thoroughly dried in an oven at 37 ℃. Subsequently, 0.1% crystal violet staining solution was added to stain the cells, and the cells were shaken for 30 minutes. The cells were washed three times with distilled water to remove excess crystal violet staining solution. Finally, the cells were thoroughly dried in an oven at 37 ℃ and then photographed for observation after drying.

2.3. Testing Knockout Efficiency

Cells were inoculated in 12-well plates, and once the cell density reached 80%, the cells were transfected with siRNA using lipo3000 transfection reagent. The final concentration of siRNA was 1 nM. After incubating for 48 hours, the medium was discarded, and Trizol reagent was added to extract the total RNA from the plates. The RNA were then reverse-transcribed using MMLV reverse transcriptase to obtain the cDNA. Subsequently, a qPCR reaction was performed to detect the expression of the relevant genes using SYBR Green dye.

2.4. Cell Proliferation Assay by Cell Counting Kit-8

Cells were inoculated in 24-well plates, and once the cell density reached 40-60%, the cells were transfected with siRNA using lipo3000 transfection reagent. The siRNA concentration was 1 nM, and the starting time of transfection was recorded as 0 hours. Subsequently, the CCK-8 cell activity assay was performed every 24 hours. For the assay, 10 μL of CCK8 reagent containing CCK8 was added to each well, followed by incubation in an incubator for 2 hours. The absorbance at 450 nm was then measured using a microplate reader.

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2.5. Colony Formation assay

For the colony formation assay, cells were plated in 6-well plates at a density of 500 cells per well and transfected. Subsequently, the cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum for a period of 10 days. Afterward, colonies were fixed using methanol, stained with 0.1% Crystal violet for 10 minutes, and photographed.

2.6. Transwell Migration Assay

A Transwell cell invasion assay was conducted using Growth Factor Reduced Matrigel invasion chambers with 8.0-mm polycarbonate filters (BD Biosciences, Shanghai, China). After transfection with the corresponding siRNA for 24 hours, the cells were inoculated into the upper layer of the chamber with Matrigel. Subsequently, 100 μL of serum-free medium was added to the upper layer, while 600 μL of complete medium containing 10% serum was added to the lower layer. The cells were then cultured in an incubator for 48 hours at 37 ℃. Following the incubation period, the medium was discarded, and the cells were washed twice with PBS. Next, the cells were fixed with formaldehyde for 30 minutes and washed twice with PBS before being subjected to crystal violet staining solution for observation.

2.7. Wound healing Assay

Cells were inoculated in 12-well plates, and once the cell density reached 80%, the cells were transfected with siRNA using lipo3000 transfection reagent. The final concentration of siRNA was 1 nM. After 24 hours of transfection, a straight line was scratched through the vertical cells using a 200-μl lance tip, and then the medium was changed to wash away the detached cells. Photos were taken at 0 hours, 24 hours, and 48 hours to observe changes in the width of the scratches over time.

3. Results and Discussion

The differentially expressed genes between tumor and normal tissues for selected candidate genes in LUAD (Table1). The gene expression profiles of differentially expressed candidates in LUAD (Figure 1). The survival curves corresponding to genes with altered expression levels in LUAD (Figure 2). The successful knockdown of selected genes was confirmed by reverse transcription followed by qPCR of gene transcripts (Figure 3 & 4).

Table 1. Differential Gene Expression Analysis

Gene Symbol	Gene ID	Median (Tumor)	Median (Normal)	Log2 (Fold Change)	adjp
DAB2IP	ENSG00000136848.16	11.87	30.099	-1.273	1.16E-84
SEMA3F	ENSG00000001617.11	12.33	37.46	-1.529	1.55E-57
AXIN2	ENSG00000168646.12	4.93	18.06	-1.684	6.12E-74

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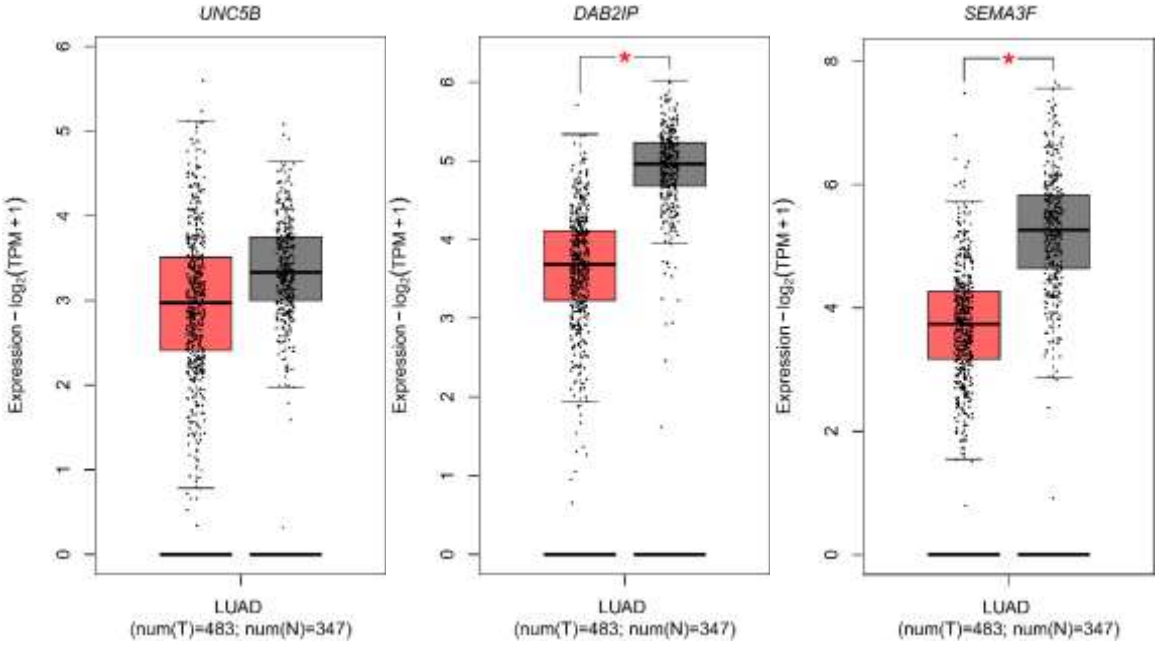

Figure 1. Gene expression profiles of 5 candidate genes in tumor tissue and normal tissue of LUAD

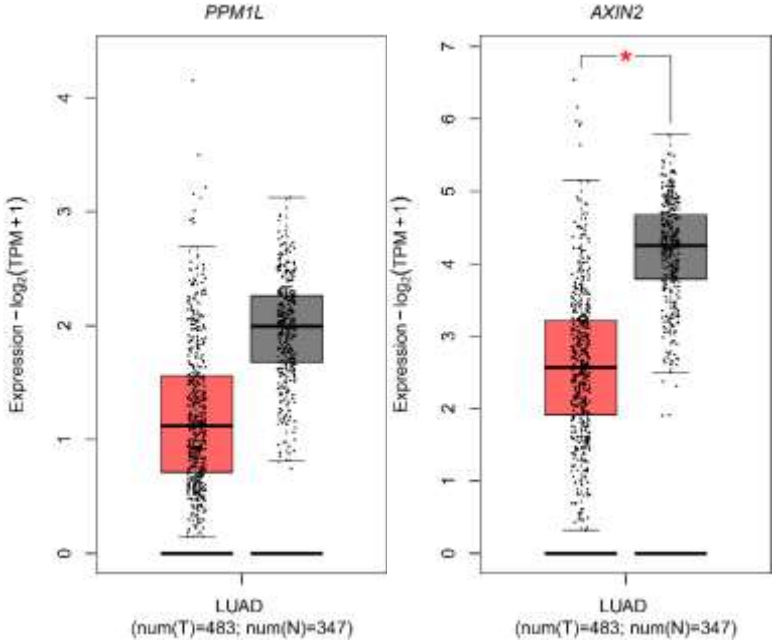

Figure 2. Kaplan-Meier survival analysis based on the expression status of one gene.

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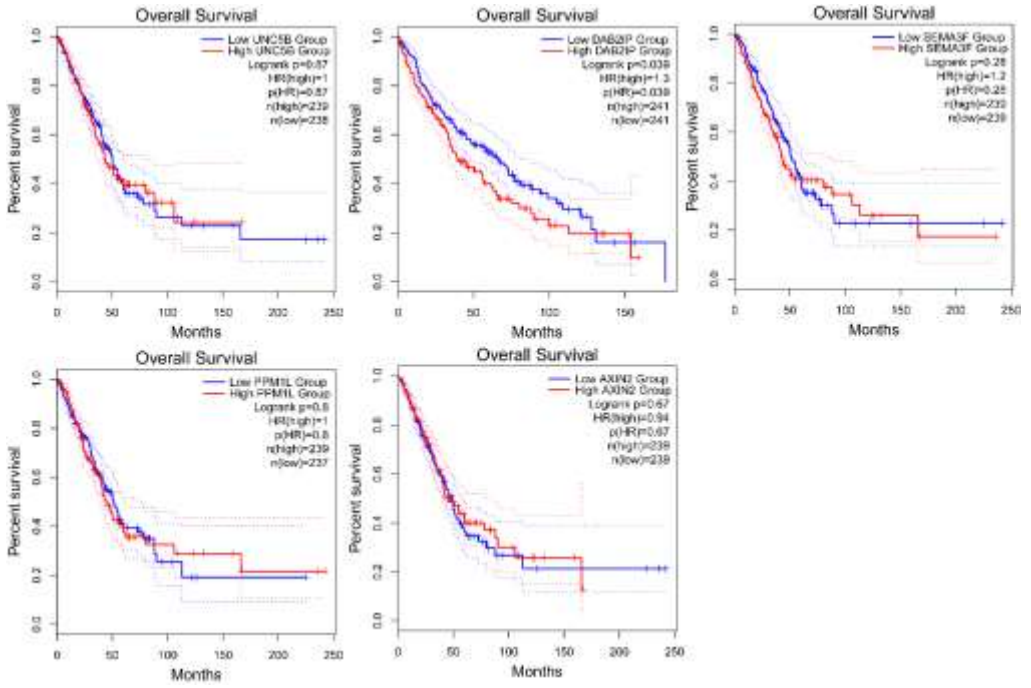

Figure 3. Measuring RNAi knockdown using qPCR.

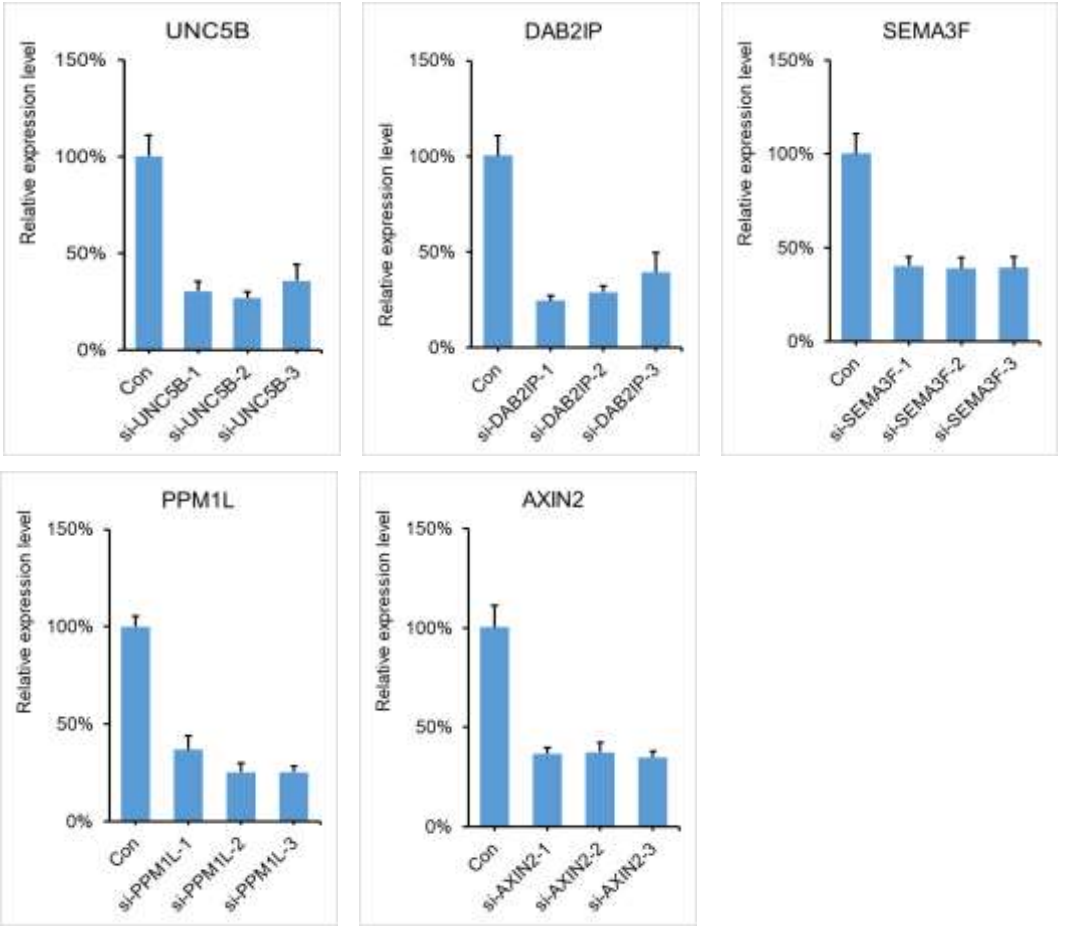

Figure 4. Gene Expression after Knockout.

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Given that genes were confirmed to be knocked down/out, we find that *DAB2IP, SEMA3F, PPM1L,* and *AXIN2* exhibit anti-tumor properties.

Cells with *DAB2IP, SEMA3F, PPM1L*, and *AXIN 2* knockdown displayed significant proliferative tendencies, as evidenced by the steeper cell growth curves (Figure 3), after knockdown of selected candidate genes, accelerated cell proliferation (Figure 5) and increased clone formation (Figure 6) were observed. The enhanced migration ability indicated by the transwell migration assay (Figure 7), and a more pronounced regenerative property demonstrated by the wound healing assay (Figure 8). However, contrary to reporting from literature and online databases, UNC5B knockdown seems to reduce proliferation, with a reduced growth curve, migration, and ability to wound-heal [10, 11]. This suggests UNC5B's role to be in contrast to its putative one: instead of being a tumor-suppressor, it seems to be important for cell proliferation and thus perhaps tumorigenesis in the case of Lung Adenocarcinoma. As such, future studies could possibly focus on the knockout of UNC5B as a therapeutic potential in the treatment of Lung Adenocarcinoma.

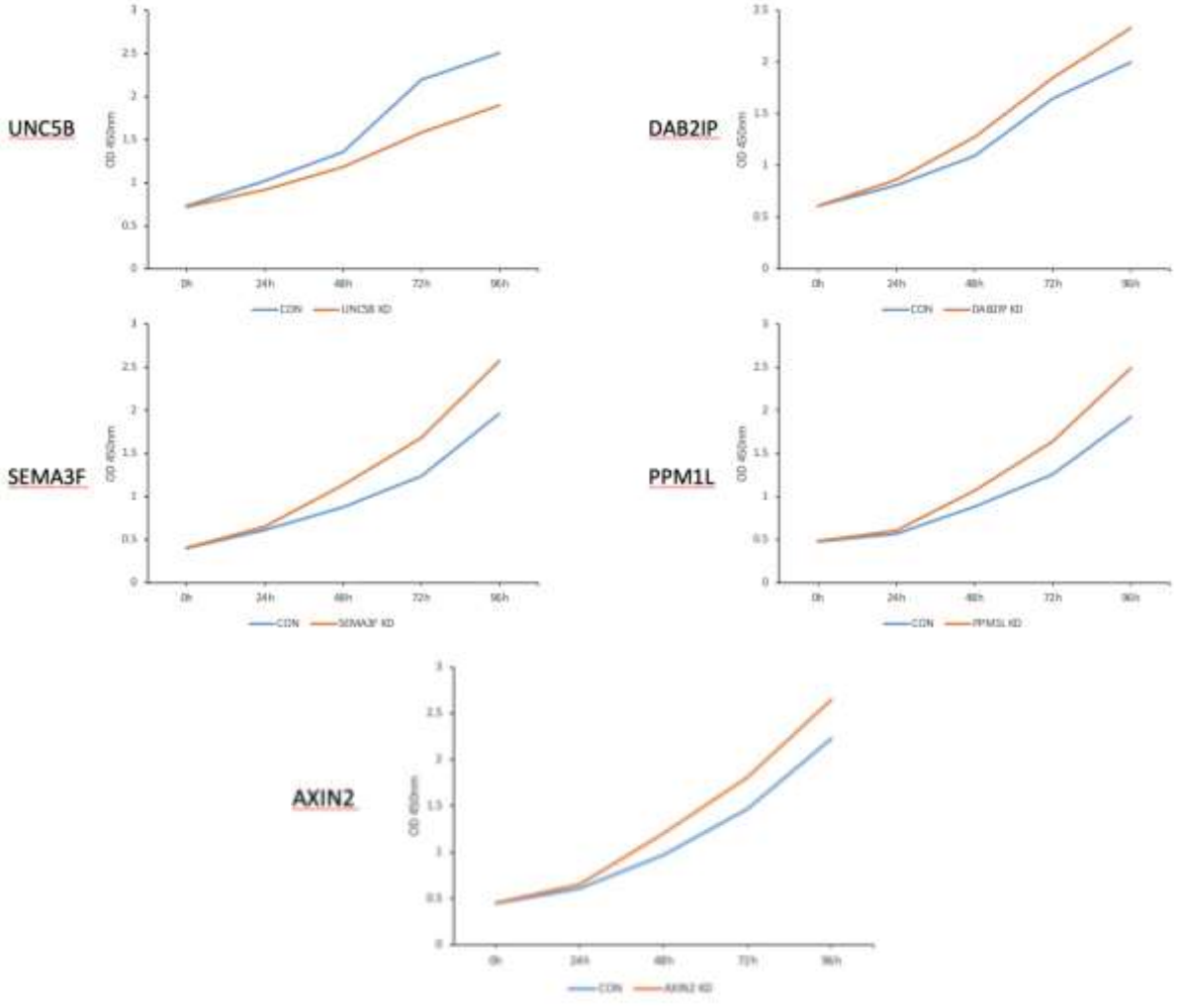

Figure 5. Cell growth curve of after knockdown.

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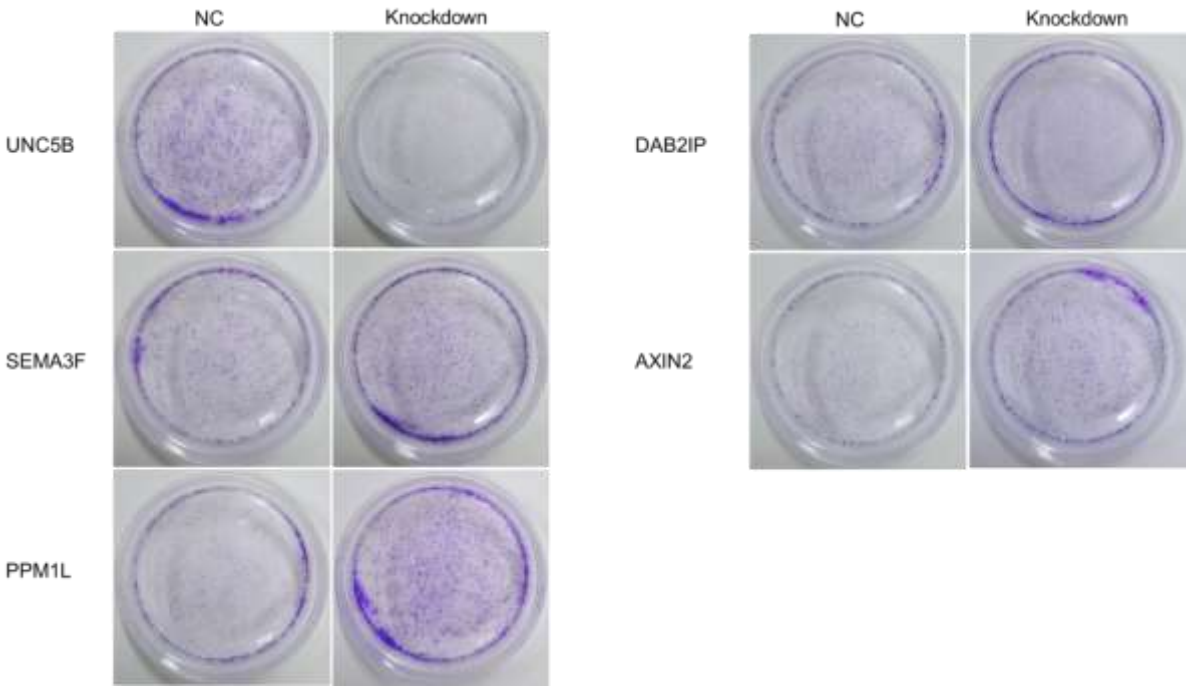

Figure 6. Cell clone formation assay after knockdown.

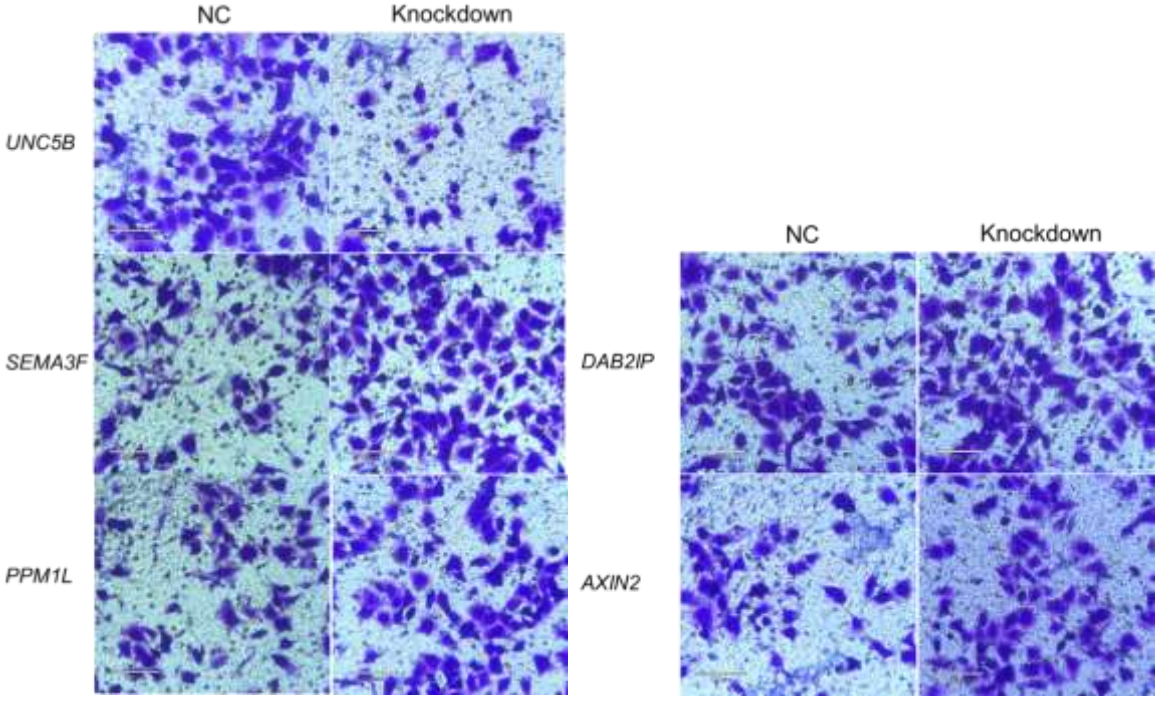

Figure 7. Transwell Migration Assay Results.

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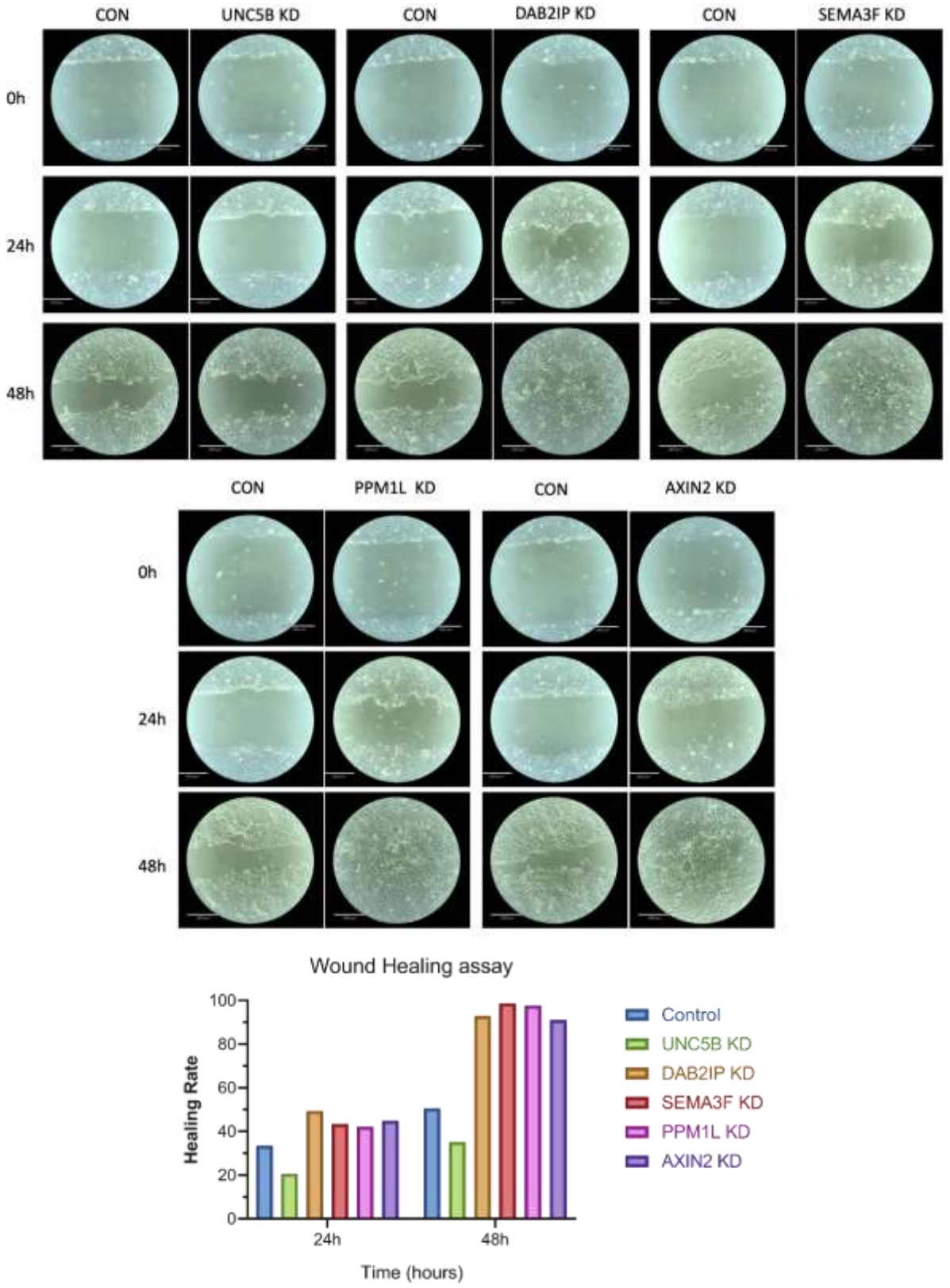

Figure 8. Healing Assay Results.

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4. Conclusion

In conclusion, our study aimed to investigate the potential tumor suppressor roles of selected candidate genes in lung adenocarcinoma (LUAD). Through the analysis of the TCGA database, we identified differentially expressed genes between tumor and normal tissues, and examined their gene expression profiles and survival curves. Furthermore, we successfully confirmed the knockdown of selected genes through reverse transcription followed by qPCR. Our results demonstrated that DAB2IP, SEMA3F, PPM1L, and AXIN2 exhibited anti-tumor properties, as evidenced by enhanced cell proliferation, increased colony formation, enhanced migration ability, and improved regenerative properties. However, contrary to previous reports, UNC5B knockdown appeared to reduce proliferation and other tumorigenic characteristics. This suggests a contrasting role for UNC5B in LUAD, indicating its potential importance for cell proliferation and tumorigenesis. Future investigations could focus on UNC5B knockout as a potential therapeutic approach for the treatment of lung adenocarcinoma. These findings contribute to a better understanding of the roles of these candidate genes in LUAD and provide insights for potential targeted therapies.

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