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Origin recognition complex subunit 1 (ORC1) augments malignant behaviors of lung adenocarcinoma cells via targeting Wnt signaling

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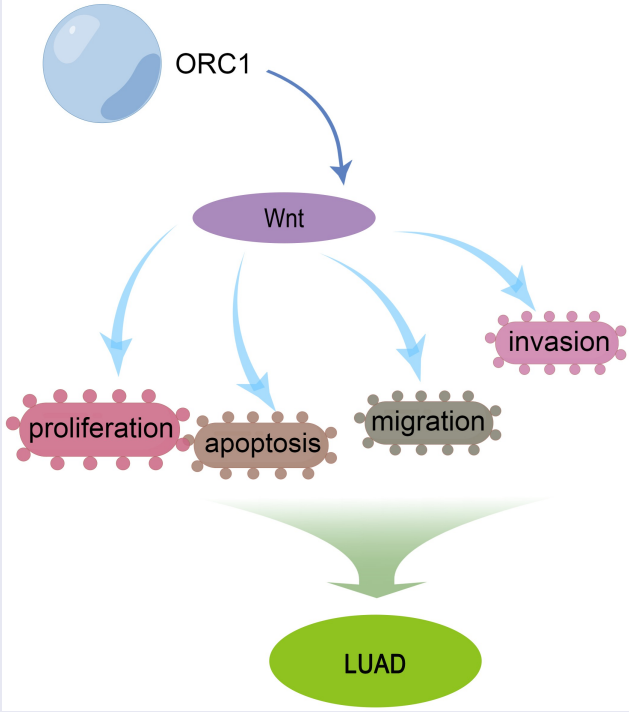
ABSTRACT

It has been reported that origin recognition complex subunit 1 (ORC1) plays an oncogenic role in certain human cancers. Nevertheless, its regulatory function in lung adenocarcinoma (LUAD) progression was poorly understood. In this study, gene and protein levels were measured via RT-qPCR and Western blotting. LUAD cell viability, apoptosis, and metastasis were determined via CCK-8, TUNEL, and Transwell assays. Bioinformatics analyses were performed using Genotype Tissue Expression (GTEx) and The Cancer Genome Atlas (TCGA) databases. Herein, it was revealed that ORC1 was evidently upregulated and positively correlated to unsatisfactory prognosis in LUAD. Besides, single-sample gene set enrichment analysis (ssGSEA) revealed that ORC1 is negatively associated with 17 immune infiltrating cells and differently expressed in several kinds of immune cells. Also, Gene Ontology (GO) analysis indicated the involvement of ORC1 in several molecular functions. In addition, *in vitro* experiments demonstrated that ORC1 facilitated malignant behaviors of LUAD cells; moreover, animal assays further affirmed that ORC1 promoted LUAD tumor growth *in vivo*. As for the molecular mechanisms involved, it was found that ORC1 depletion inhibited the Wnt pathway in LUAD cells. Furthermore, rescue experiments demonstrated that Wnt signaling activation could abate the impacts of ORC1 knockdown on tumorigenic phenotypes of LUAD cells. In conclusion, our findings demonstrated that ORC1 promoted LUAD progression by regulating the Wnt signaling, indicating ORC1 could be an auspicious biomarker or target for LUAD diagnosis and treatment.



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Highlights

- ORC1 is upregulated and of certain prognostic significance in LUAD.
- ORC1 is associated with immune infiltration in LUAD.
- ORC1 increases proliferation and metastasis but reduces apoptosis in LUAD cells.
- ORC1 aggravates malignant behaviors of LUAD cells by activating Wnt signaling.
- ORC1 facilitates LUAD tumor growth.

Introduction

Currently, lung cancer is a common and fatal human cancer globally [1]. Non-small cell lung cancer (NSCLC) makes up 85% of newly diagnosed lung cancer cases every year [2]. As the most prevalent subtype of NSCLC [3], lung adenocarcinoma (LUAD) is featured with a high mortality rate and poor prognosis [4]. Due to atypical clinical symptoms at early stages and inefficient screening methods, most LUAD patients are diagnosed at advanced stages [5]. Despite great achievements made in recent years, the efficacies of current therapies for LUAD are still limited [6]. The average 5-year survival rate for LUAD patients remains below 10% [7]. Therefore, it is extremely important to explore latent molecular mechanisms involved in LUAD.

Origin-recognition complex proteins (ORCs), the central component of pre-replication complex in eukaryotes, are essential to the maintenance of genomic stability [8–10]. Among the six ORC subunits (ORC1-6), origin-recognition complex subunit 1 (ORC1) is closely involved in DNA replication and organ development [11]. As a functional protein, ORC1 plays a fundamental role in modulating cellular processes, including proliferation, apoptosis, and cell cycle [12–14]. Recently, accumulating evidence indicates that ORC1 dysregulation is associated with the tumorigenesis of human cancers, including triple-negative breast cancer [15], cervical cancer [16], and tongue squamous cell carcinoma [17], indicating it could be a regulatory protein in human malignancies. Hence, we presumed that ORC1 may also be a functional regulator in LUAD.

In our study, we intended to investigate the functional role and potential mechanism of ORC1 in LUAD. Based on bioinformatics analyses, we detected ORC1 expression in LUAD, evaluated its prognostic and diagnostic values, and explored the association between ORC1 expression and immune infiltration as well as biological functions in LUAD. Then, *in vitro* and *in vivo* experiments were performed to verify ORC1 expression and functions in LUAD. Also, the downstream signaling pathway of ORC1 in LUAD was screened and confirmed. Our findings elaborated that ORC1 contributed to LUAD progression via activating the Wnt signaling, indicating that ORC1 exerted essential effects on LUAD development and might be a promising target for LUAD therapy.

Materials and methods

Cell culture and transfection

Human LUAD cell lines (ABC-1, H358, and H1435) and normal bronchial epithelial cell line (BEAS-2B) were acquired from BeNa Culture Collection (Beijing, China) and cultivated in a humidified incubator (RPMI 1640 + 10% FBS; 5% CO_2; 37°C).

Small interfering RNA (siRNA) of ORC1 (si-ORC1) and negative control (si-NC), ORC1 pcDNA 3.1 vector (oe-ORC1), and pcDNA 3.1 empty vector (Vector) were transfected into H358 or H1435 cells via Lipofectamine 3000 (Invitrogen, USA) and incubated for 24 h before experiments.

RT-qPCR

TRIzol reagent (Invitrogen, USA) was used to extract total RNAs from cells. Then, cDNA was reversely transcribed from total RNA via RNA PCR Kit (Takara, China). Then, gene expression levels were detected via qPCR performed in StepOnePlus™ Real-Time PCR System (Applied Biosystems) with SYBR Green PCR Master Mix (Invitrogen) and normalized to GAPDH by $2^{-\Delta\Delta Ct}$ method. The following primers were used: ORC1 Forward: 5′-CTCAAGCCT AGAACGCCACGTT-3′, Reverse 5′-GGAAGAGAC

TCAGGTACAGCAG-3′; GAPDH Forward: 5′-GTCTCCTCTGACTTCAACAGCG-3′ Reverse: 5′-ACCACCCTGTTGCTGTAGCCAA-3′.

Western blotting

Lysis buffer was utilized to extract protein samples from cells. Then, protein samples were separated by SDS-PAGE and transferred to PVDF membranes. Next, the membranes were blocked with 5% skim milk for 2 h, probed with primary antibodies (anti-β-catenin, anti-cyclin D1, anti-c-myc, and anti-ORC1) at 4°C overnight, and incubated with secondary antibodies at room temperature. Subsequently, the protein bands were visualized with ECL (Millipore), with GAPDH as the internal control.

CCK-8

LUAD cells were transferred to 96-well plates (2×10^3 cells/well). After LUAD cells were cultivated for the indicated time, CCK-8 reagent (10 μl/well) was added. 2 hours later, the absorbance (OD value) at 450 nm was detected with a microplate reader (Bio-Rad, USA).

TUNEL assay

Via the Cell Death Detection Kit (Roche, USA), TUNEL assay was performed to detect cell apoptosis as per standard protocol. Briefly, treated LUAD cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton-X 100, incubated TUNEL reaction mixture (In Situ Cell Detection Kit, Roche Diagnostics), and then stained with DAPI. TUNEL-positive cells were counted with a fluorescent microscope (Nikon) to calculate the apoptotic rate of LUAD cells.

Animal models

Male BALB/c nude mice (6 weeks old) were purchased from Shanghai SLAC Laboratory Animal, Co., Ltd. (Shanghai, China). In brief, H358 cells (2×10^6) transfected with si-NC and si-ORC1 were subcutaneously inoculated into the right flank of the mice, respectively. The tumor volume was measured every 5 days. 30 days later, all mice were sacrificed by cervical dislocation to harvest tumors. Finally, tumors were weighed. The animal study was approved by the Ethics Committee of Xuzhou Cancer Hospital.

Transwell assay

Transwell chambers (Corning, USA) (polycarbonate membrane coated with or without Matrigel) were applied for cell invasion or migration detection. The upper compartment contained serum-free medium; the lower compartment contained complete medium. In brief, treated LUAD cells (1×10^5) were seeded into the upper compartment. After 24 h's incubation, the invaded or migrated cells on the lower surface of the membrane were fixed, stained, and finally counted under the microscope (Thermo Fisher Scientific).

Statistical analysis

All data analyses were conducted using SPSS software. All experiments were repeated 3 times and the results were displayed as mean ± standard deviation (SD). Differences between groups were determined via Student's t-test, and differences among multi-groups were analyzed using one-way or two-way ANOVA followed by Tukey's post hoc test. The chi-square test was used to analyze the association between ORC1 expression and clinicopathological features of LUAD patients. The Kaplan-Meier method and log-rank test were performed to determine the overall survival rates and the survival curve, and the median expression of ORC1 was used as the cutoff value. The receiver operating characteristic (ROC) curve was used to analyze the diagnostic value of ORC1 expression. P-value < 0.05 was deemed significant in statistics.

Results

First of all, bioinformatics analyses were performed to investigate ORC1 expression, its prognostic and diagnostic values, the association between ORC1 and immune infiltration, and the biological functions of ORC1 co-expressive genes. Next, *in vitro* and *in vivo* experiments were performed to demonstrate the functional role of ORC1 in LUAD tumorigenesis. Finally,

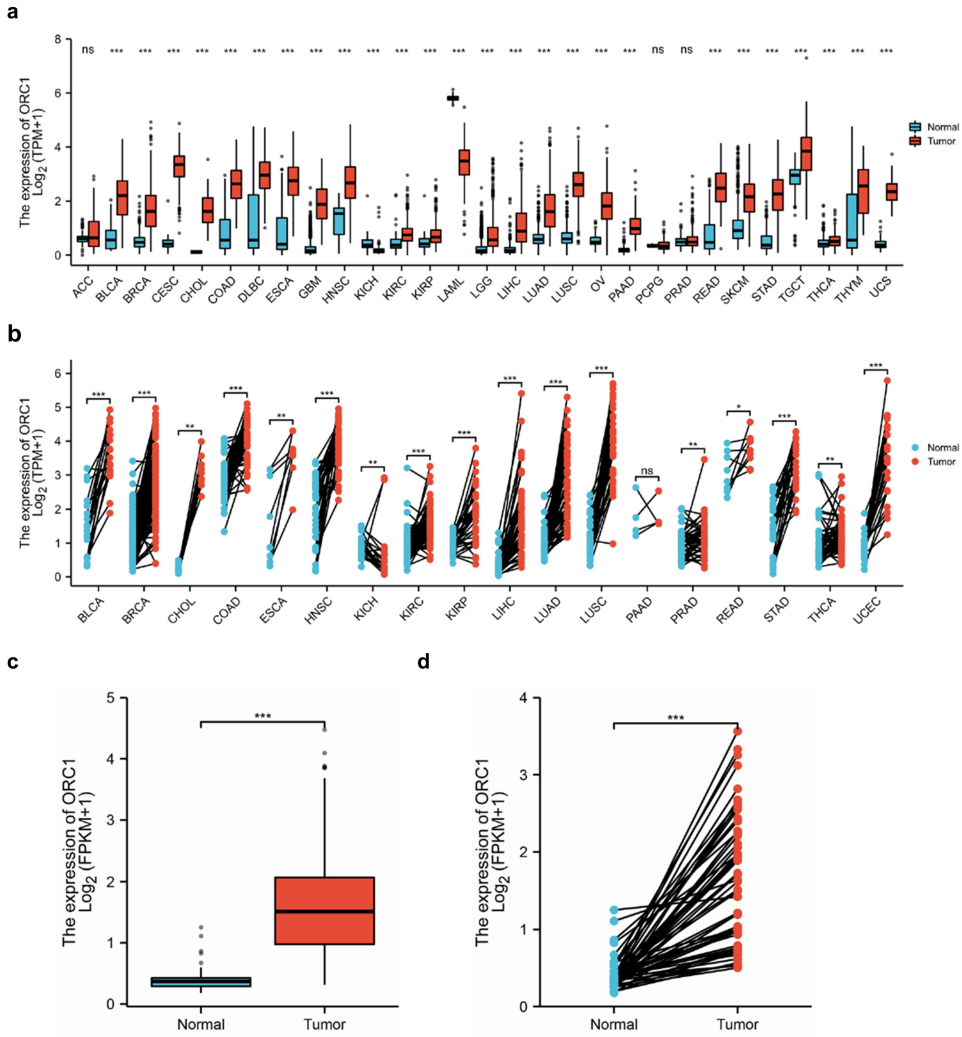

Figure 1. Upregulated ORC1 expression in LUAD. (a) ORC1 level in tumor tissues and normal tissues based on GTEx and TCGA databases. ORC1 is significantly upregulated in BLCA ($P < 0.001$), BRCA ($P < 0.001$), CESC ($P < 0.001$), ($P < 0.001$), COAD ($P < 0.001$), DLBC ($P < 0.001$), ESCA ($P < 0.001$), GBM ($P < 0.001$), HNSC ($P < 0.001$), KIRC ($P < 0.001$), KIRP ($P < 0.001$), LGG ($P < 0.001$), LIHC ($P < 0.001$), LUAD ($P < 0.001$), LUSC ($P < 0.001$), OV ($P < 0.001$), PAAD ($P < 0.001$), READ ($P < 0.001$), SKCM ($P < 0.001$), STAD ($P < 0.001$), TGCT ($P < 0.001$), THCA ($P < 0.001$), THYM ($P < 0.001$) and UCS ($P < 0.001$) but significantly downregulated in KICH ($P < 0.001$) and LAML ($P < 0.001$). Besides, ORC1 is relatively upregulated in ACC ($P = 0.342$) but downregulated in PCPG ($P = 0.679$; with no statistical significance) and PRAD ($P = 0.155$; with no statistical significance). (b) ORC1 level in LUAD and adjacent normal tissues based on TCGA database. ORC1 is significantly upregulated in BLCA ($P < 0.001$), BRCA ($P < 0.001$), CHOL ($P = 0.004$), COAD ($P < 0.001$), ESCA ($P = 0.008$), HNSC ($P < 0.001$), KIRC ($P < 0.001$), KIRP ($P < 0.001$), LIHC ($P < 0.001$), LUAD ($P < 0.001$), LUSC ($P < 0.001$), PRAD ($P = 0.005$), READ ($P = 0.027$), STAD ($P < 0.001$), THCA ($P = 0.004$) and UCEC ($P < 0.001$) but significantly downregulated in KICH ($P = 0.005$). Besides, ORC1 is relatively upregulated in PAAD ($P = 0.875$; with no statistical significance). (c) ORC1 level in LUAD and normal tissues based on TCGA-LUAD dataset. (d) ORC1 level in LUAD and paired normal tissues based on TCGA-LUAD dataset. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

the downstream mechanism of ORC1 in LUAD was explored.

Upregulated ORC1 expression in LUAD

ORC1 has been reported to be upregulated in various malignancies [15–17]. To investigate the gene expression profile of ORC1 in human cancers, we analyzed ORC1 levels in tumor tissues and normal tissues according to UCSC XENA human RNA-seq gene expression data (https://xenabrowser.net/datapages/) based on Genotype Tissue Expression (GTEx) and The Cancer Genome Atlas (TCGA) databases [18]. As

shown in Figure 1(a), ORC1 level was upregulated in a variety of human cancers, including LUAD. Then, ORC1 levels in cancer tissues and adjacent normal tissues were analyzed, and it was shown that ORC1 level was elevated in a variety of human cancers (Figure 1(b)). ORC1 was highly expressed in LUAD tissues, relative to normal tissues (Figure 1(c)); in addition, higher ORC1 expression was observed in LUAD tissues, contrasted with paired normal tissues (Figure 1(d)). The above results suggested that ORC1 level might be oncogenic in LUAD.

Prognostic significance of ORC1 in LUAD

To investigate the association between ORC1 and LUAD prognosis and progression, clinicopathological data from TCGA-LUAD dataset was analyzed. Firstly, the Kaplan-Meier method was used to determine the effect of ORC1 expression on the survival of LUAD patients. Intriguingly, it was found that high ORC1 expression was correlative to unsatisfactory overall survival (OS) (Figure 2(a)), disease-specific survival (DSS) (Figure 2(b)), and progress-free interval (PFI) (Figure 2(c)) of LUAD patients. Through further analysis, it was also found that ORC1 expression was remarkably associated with LUAD grading and staging (Figure 2(d-g), Table 1), implying ORC1 could be a potential indicator for LUAD progression. To further analyze the diagnostic significance of ORC1, the ROC curve of ORC1 in LUAD was plotted based on TCGA-LUAD dataset (Figure 2(h)). The area under the curve (AUC) was 0.961, suggesting ORC1 might be a latent biomarker for LUAD diagnosis. To sum up, ORC1 might be of certain prognostic value in LUAD.

ORC1 is associated with immune infiltration in LUAD

High immune cell infiltration indicated a favorable prognosis in LUAD [19]. By single-sample gene set enrichment analysis (ssGSEA), we analyzed the relationship between ORC1 expression and the abundance of 24 immune infiltrates based on TCGA-LUAD dataset. It was found that ORC1 expression was negatively correlated with 17 immune infiltrates, including Mast cells, iDC, Eosinophils, TFH, DC, CD8 T cells (Figure 3(a)). Further research showed significant differences in

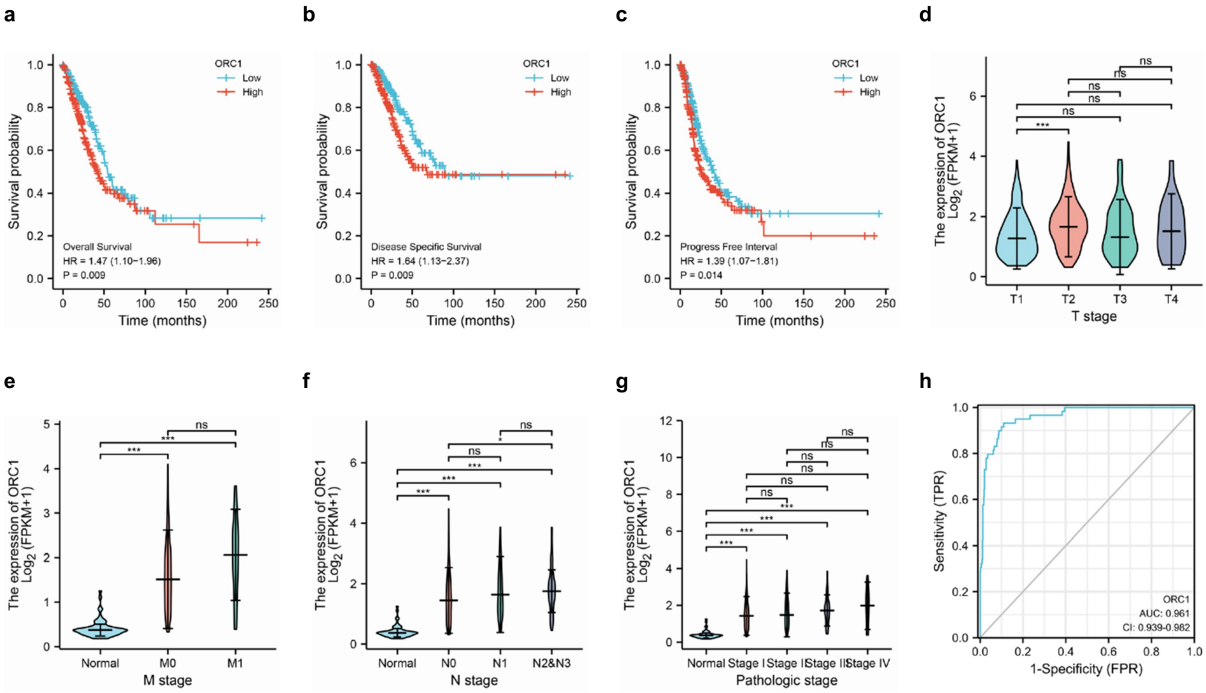

Figure 2. Prognostic significance of ORC1 in LUAD. (a-c) OS (a), DSS (b), and PFI (c) curves of ORC1 based on TCGA-LUAD dataset. (d-g) Correlation between ORC1 expression and T stage (d), M stage (e), N stage (f), as well as pathologic stage (g) based on TCGA-LUAD dataset. (h) ROC curve of ORC1 based on TCGA-LUAD dataset; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 1. Association between ORC1 expression and clinicopathological features of LUAD patients.

Characteristic	ORC1		*p* value
	Low expression (n = 267)	High expression (n = 268)	
Age, median (IQR)	67 (60, 74)	65 (58, 71)	0.021
T stage, n (%)			< 0.001
T1	109 (20.5%)	66 (12.4%)	
T2	119 (22.4%)	170 (32%)	
T3	28 (5.3%)	21 (3.9%)	
T4	9 (1.7%)	10 (1.9%)	
N stage, n (%)			0.025
N0	181 (34.9%)	167 (32.2%)	
N1	47 (9.1%)	48 (9.2%)	
N2	25 (4.8%)	49 (9.4%)	
N3	1 (0.2%)	1 (0.2%)	
M stage, n (%)			0.022
M0	180 (46.6%)	181 (46.9%)	
M1	6 (1.6%)	19 (4.9%)	

ORC1 level among the immune infiltrates, including aDC, B cells, CD8 T cells, Th2 cells, and so on (Figure 3(b)). Taken together, ORC1 might play a critical role in modulating immune functions in LUAD.

Identification of biological functions of ORC1 co-expressive genes

To further probe the biological function of ORC1 in LUAD, ORC1-related gene expression profiling analysis was performed. A total of 394 upregulated genes and 446 downregulated genes were considered to be significantly associated with ORC1 expression ($log_2FC > 2$ or < -2; Padj < 0.01) (Figure 4(a)). Among them, top 30 dysregulated genes were shown in Figure 4(b). Furthermore, GO enrichment analysis was also performed, and as shown in Figure 4(c), molecular function terms associated with ORC1-associated genes included axon terminus, neuron projection terminus, synaptic vesicle cycle, and so on.

ORC1 increases proliferation and reduces apoptosis in LUAD cells

According to RT-qPCR and Western blotting results, ORC1 level was largely upregulated in LUAD cell lines (ABC-1, H358, and H1435), relative to normal cell line (BEAS-2B) (Figure 5(a)), thereby affirming ORC1 was highly expressed in LUAD. As higher ORC1 levels were observed in H358 and H1435 cells, the two cell lines were used for functional assays. Enhanced proliferation and inhibited apoptosis are typical features of almost all cancer cells, including LUAD cells [20]. To explore the biological functions of ORC1 in regulating LUAD cell proliferation and apoptosis, H358 and H1435 cells were stably transfected with si-NC, si-ORC1, Vector, or oe-ORC1. The knockdown and overexpression efficiency of si-ORC1 and oe-ORC1 were confirmed by RT-qPCR and western blot assays (Figure 5(b,c)). CCK-8 assay manifested that lower ORC1 level inhibited the proliferation efficiency of H358 and H1435 cells, while higher ORC1 level exerted an opposite effect (Figure 5(d)). Moreover, TUNEL assay showed that ORC1 knockdown largely enhanced the apoptotic ratio of LUAD cells; on the contrary, ORC1 overexpression inhibited LUAD cell apoptosis (Figure 5(e,f)). These results revealed that ORC1 might promote cell proliferation and reduce apoptosis in LUAD.

ORC1 facilitates LUAD cell migration and invasion

As demonstrated by recent studies, strong invasiveness and metastasis are distinctive features of LUAD [21]. To analyze the impacts of ORC1 on the migrative and invasive capacities of LUAD cells. Transwell assays were conducted on H358 and H1435 cells transfected with si-NC, si-ORC1, Vector, or oe-ORC1. As shown in Figure 6(a,b), ORC1 overexpression significantly enhanced LUAD cell migration and invasion, whereas ORC1 silencing markedly diminished the migrative and invasive capabilities of LUAD cells. The above results suggested that ORC1 promoted LUAD cell metastasis.

ORC1 activates Wnt signaling in LUAD *in vitro*

To inquire into the potential ORC1-related signaling pathways, Gene Set Enrichment Analysis (GSEA) was employed to analyze data acquired from TCGA database. WNT signaling pathway was significantly associated with ORC1 high expression in LUAD (Figure 7(a)). Previous studies have demonstrated that Wnt signaling plays a crucial part in LUAD tumorigenesis [22].

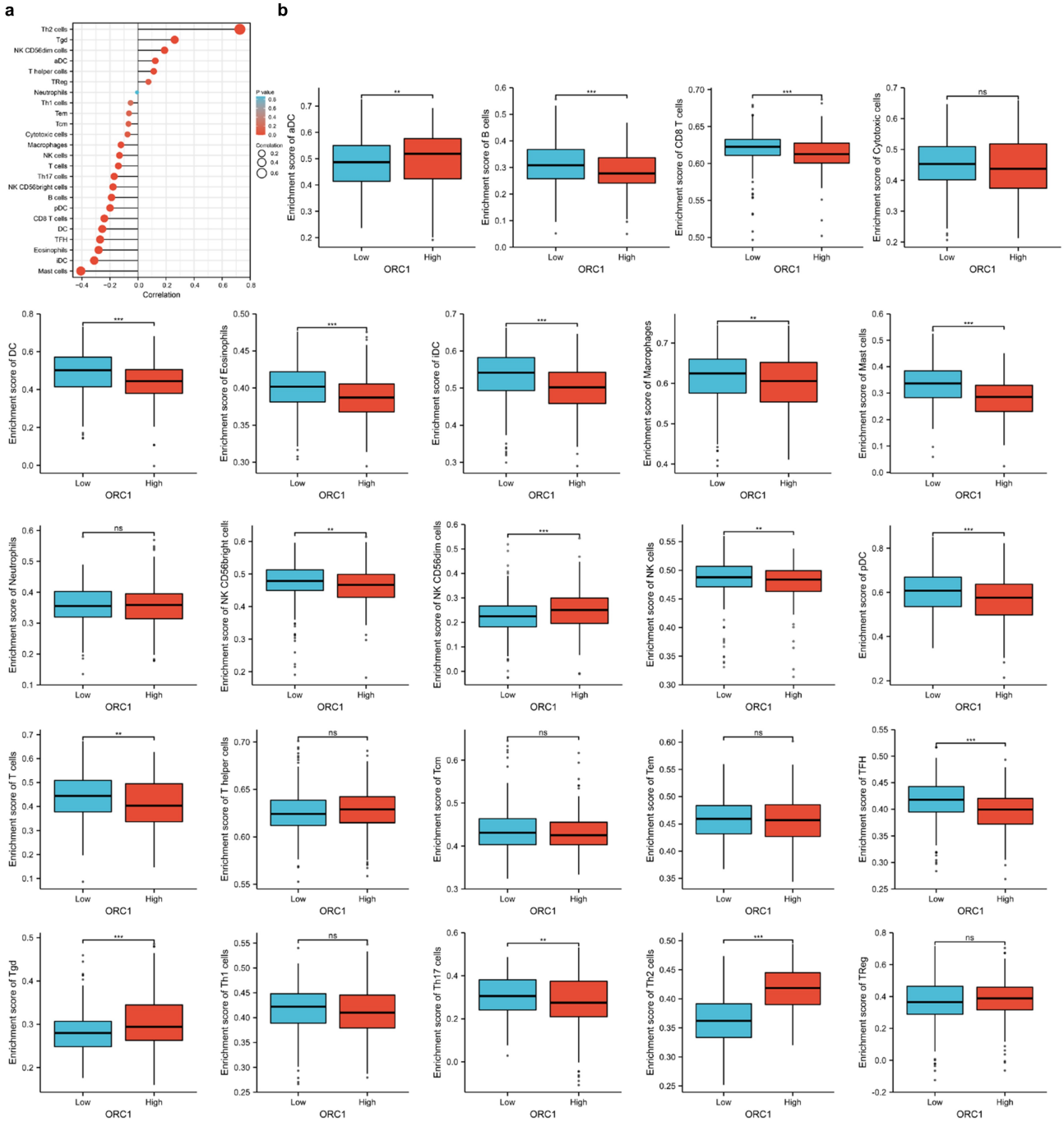

Figure 3. ORC1 is associated with immune infiltration in LUAD. (a) Relationship between ORC1 expression and immune cells. (b) Enrichment differences between high and low ORC1 expression groups in different immune cells. n = 3. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Therefore, we further investigated the relationship between ORC1 and Wnt signaling in LUAD. First of all, si-NC, si-ORC1, Vector, or oe-ORC1 was transfected into H358 and H1435 cells. Then, levels of related proteins (c-myc, cyclin D1, and β-catenin) were detected via western blotting. Our results manifested that ORC1 deletion significantly reduced c-myc, cyclin D1, and β-catenin levels, whereas ORC1 overexpression upregulated the protein levels (Figure 7(b,c)), suggesting ORC1 might activate Wnt signaling in LUAD cells. BML-284 (10 μM), a selective Wnt activator, was widely used to activate the Wnt signaling in former studies [23,24]. As indicated by western

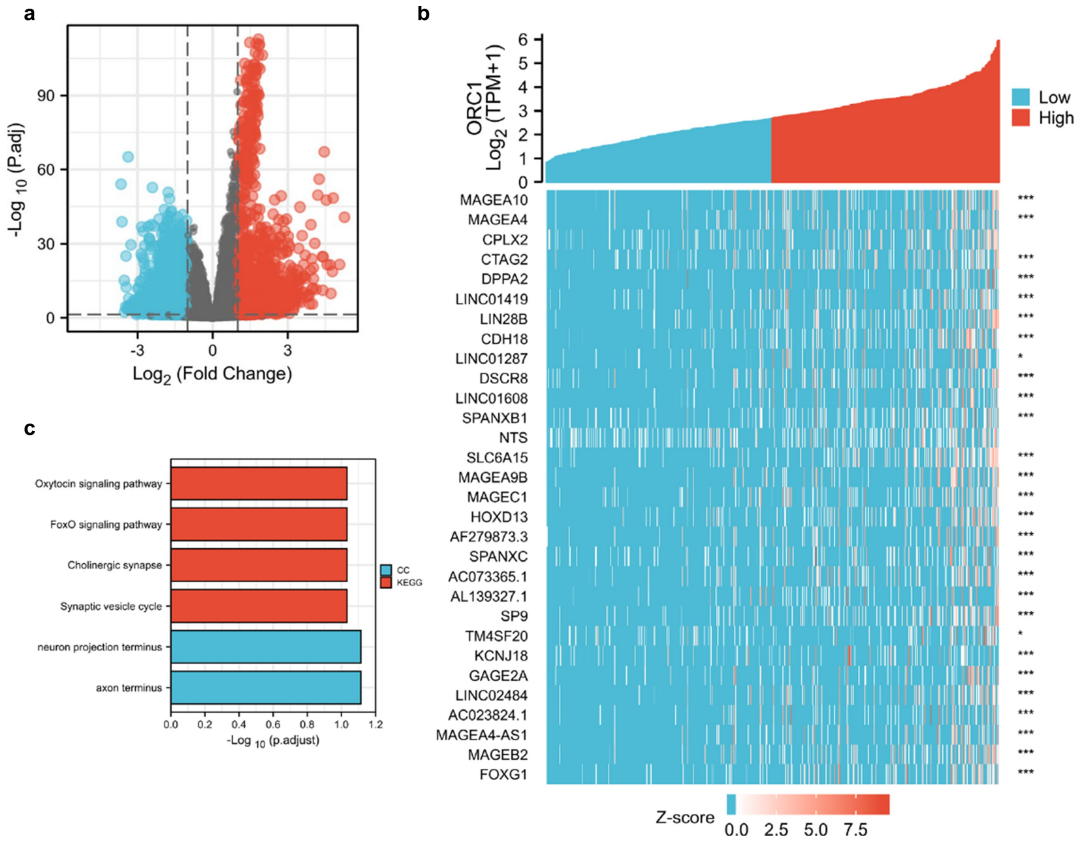

Figure 4. Identification of biological functions of ORC1 co-expressive genes. (a) Volcanic plot of differentially expressed genes based on ORC1 expression. (b) Heat map showing 30 top upregulated or downregulated genes based on ORC1 expression. (c) GO enrichment analysis results of 30 top upregulated or downregulated genes based on ORC1 expression. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

blotting assay, BML-284 partly abated the deactivation of Wnt signaling in H358 and H1435 cells induced by ORC1 knockdown (Figure 7(d)). Therefore, ORC1 promoted the actuation of Wnt signaling in LUAD cells.

ORC1 aggravates malignant cellular behaviors through Wnt signaling in LUAD

Next, we probed into the correlation between ORC1 level and Wnt activation in LUAD progression *in vitro*. According to the results of rescue experiments, BML-284-mediated stimulation of Wnt signaling dramatically reversed the repressive effects on LUAD cell viability, migration, and invasion, along with the promoting effect on LUAD cell apoptosis caused by ORC1 deletion (Figure 8(a-e)). To sum up, ORC1 conferred malignant phenotypes in LUAD in vitro via regulating the Wnt pathway.

ORC1 facilitates LUAD tumor growth

To further affirm the role of ORC1 in LUAD tumorigenesis *in vivo*, we established xenograft tumor models based on H358 cells transfected with si-NC or si-ORC1. It was found that ORC1 silencing reduced the volume and weight of xenograft LUAD tumors (Figure 9(a,b)). Furthermore, IHC analysis confirmed that si-ORC1 group exhibited lower Ki-67 expression compared to si-NC group (Figure 9(c)). All these results suggested that ORC1 promotes LUAD tumor growth *in vivo*.

Discussion

As one of the most lethal malignancies, LUAD poses a serious threat to people's life and health [25]. Since LUAD is a multifactorial and heterogeneous cancer with various genetic mutations [26], its pathogenesis is extremely complex. At present, there are still no clinically effective

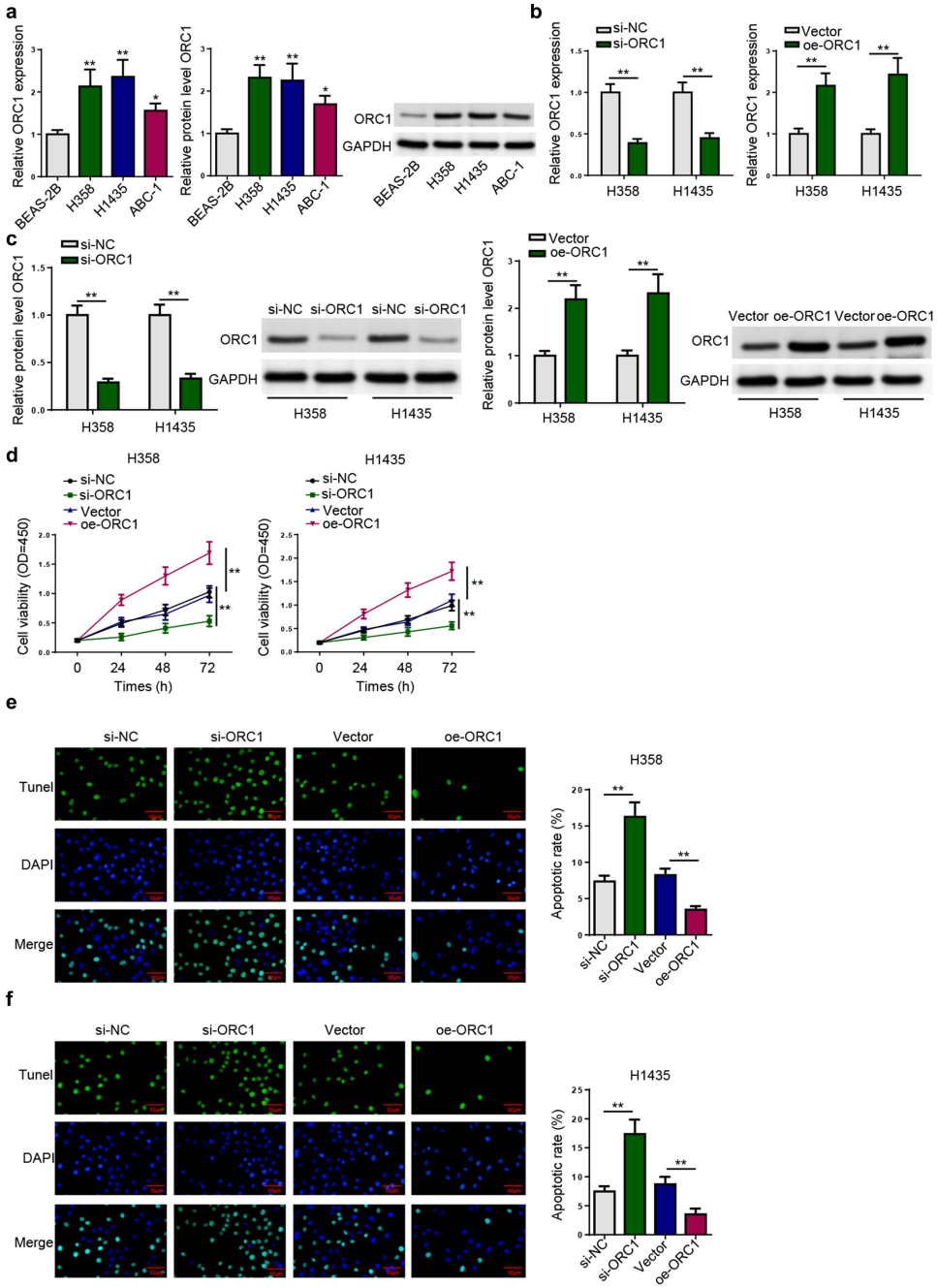

Figure 5. ORC1 increases proliferation and reduces apoptosis in LUAD cells. (a) ORC1 mRNA and protein levels in LUAD cell lines (ABC-1, H358, and H1435) and normal cell line (BEAS-2B) were determined by RT-qPCR and Western blotting. (b) ORC1 mRNA levels in si-NC, si-ORC1, Vector, or oe-ORC1 transfected H358 and H1435 cells were determined by RT-qPCR. (c) ORC1 protein levels in si-NC, si-ORC1, Vector, or oe-ORC1 transfected H358 and H1435 cells were determined by Western blotting. (d) Cell viability of si-NC, si-ORC1, Vector, or oe-ORC1 transfected H358 and H1435 cells were detected by CCK-8 assay (e and f) Cell apoptosis of si-NC, si-ORC1, Vector, or oe-ORC1 transfected H358 and H1435 cells were detected by TUNEL. n = 3. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

diagnostic and therapeutic methods for LUAD patients [27]. Therefore, revealing potential mechanisms in LUAD pathogenesis will help to find novel biomarkers and targets for LUAD treatment. Former studies have identified that ORC1 is upregulated in human cancers and facilitates cancer progression. To cite an instance, ORC1 regulates glioma cell growth and metastasis by stimulating the ERK/JNK signaling [28]. Besides, upregulation of ORC1 accelerates cervical cancer progression by promoting cell proliferation and reducing cell

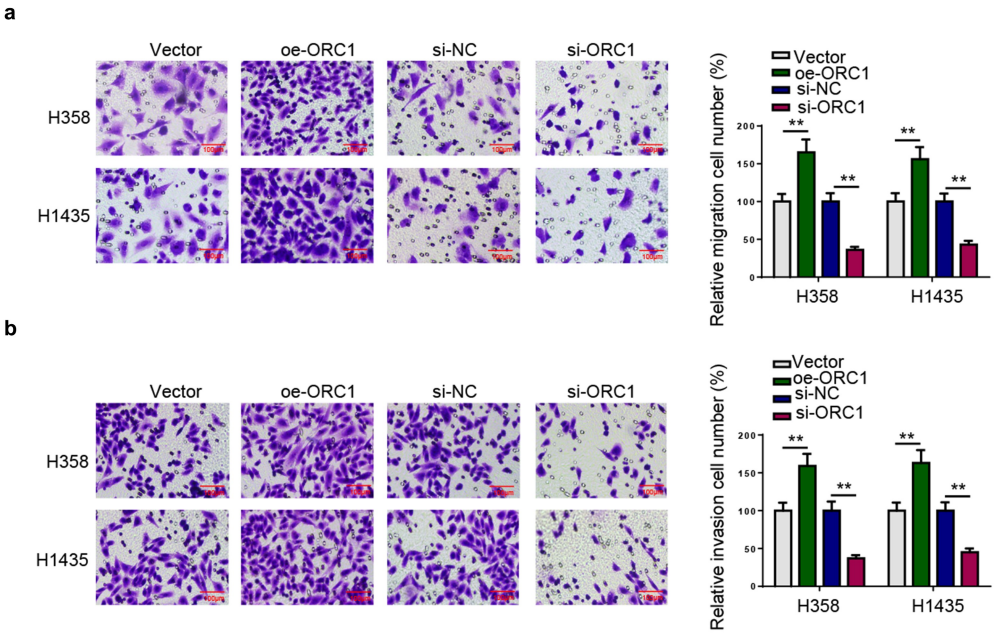

Figure 6. ORC1 facilitates LUAD cell migration and invasion. (a and b) Transwell assays were performed to detect migration and invasion of si-NC, si-ORC1, Vector, or oe-ORC1 transfected H358 and H1435 cells. n = 3. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

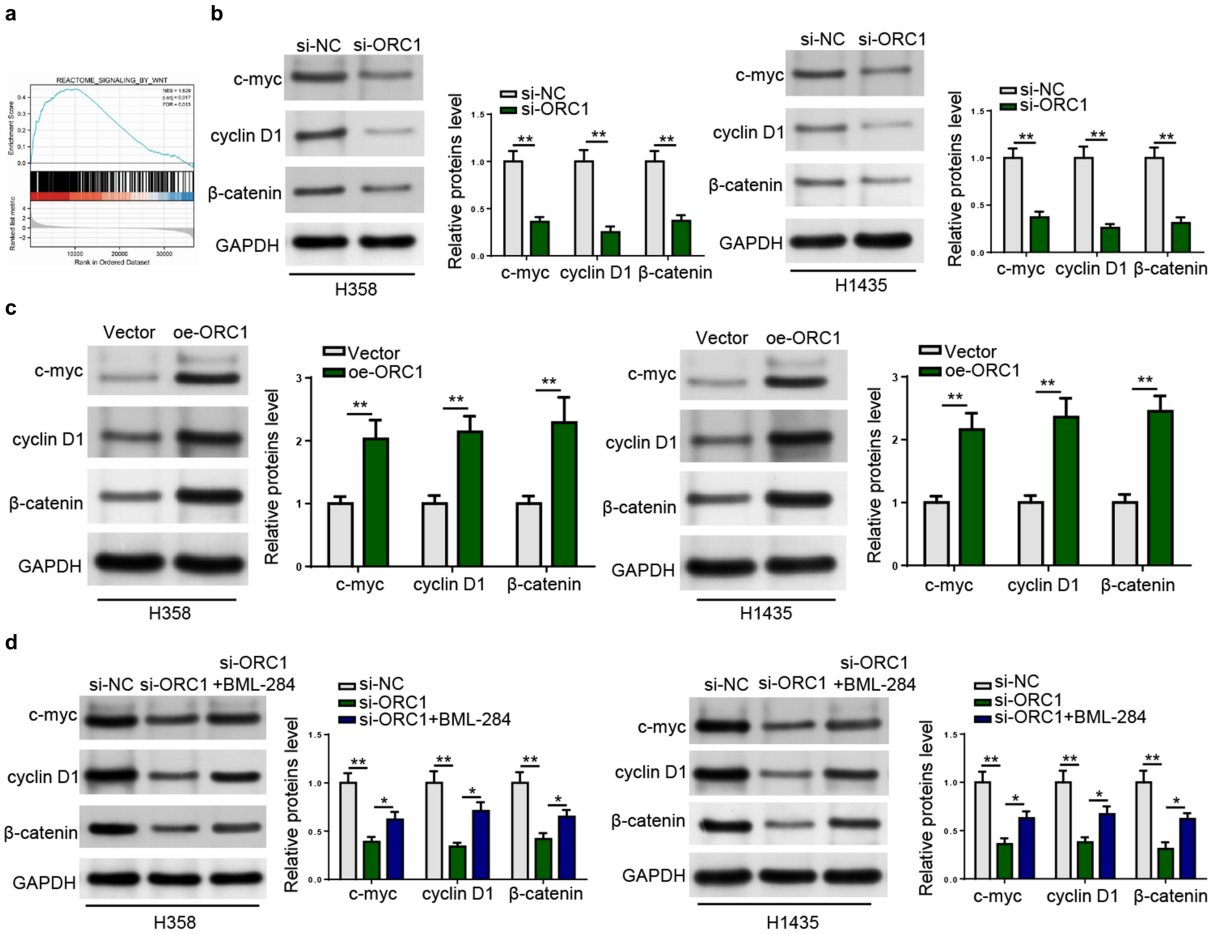

Figure 7. ORC1 activates Wnt signaling in LUAD *in vitro*. (a) Enrichment analysis of ORC1 in Wnt signaling pathway by GSEA. (b) β-catenin, cyclin D1, and c-myc levels in si-NC or si-ORC1 transfected H358 and H1435 cells were determined by Western Blotting. (c) β-catenin, cyclin D1, and c-myc levels in Vector or oe-ORC1 transfected H358 and H1435 cells were determined by Western Blotting. (d) H358 and H1435 cells were divided into si-NC, si-ORC1, and si-ORC1+ BML-284 (10 μM) groups. β-catenin, cyclin D1, and c-myc levels in H358 and H1435 cells from each group were determined by Western Blotting. n = 3. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

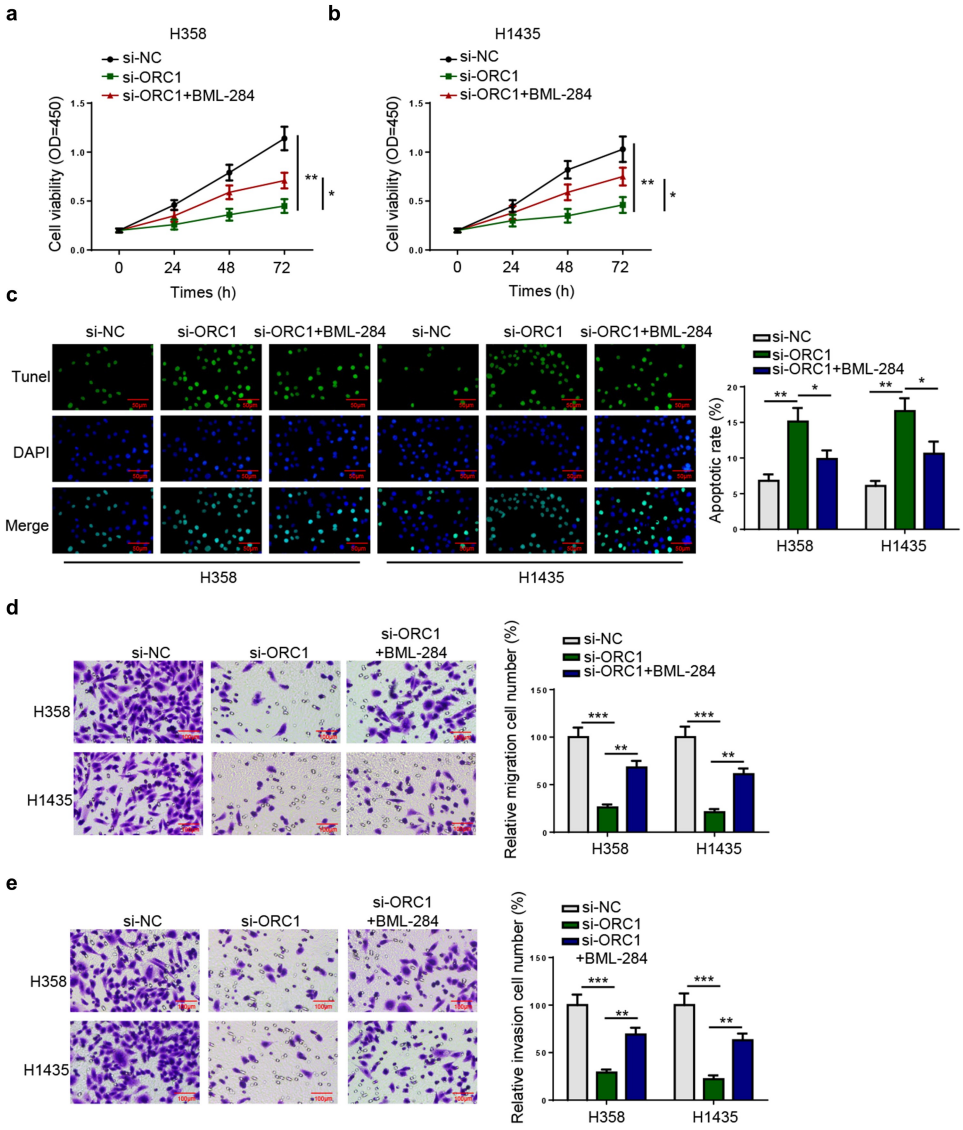

Figure 8. ORC1 aggravates malignant cellular behaviors through Wnt signaling in LUAD. (a-e) H358 and H1435 cells were divided into si-NC, si-ORC1, and si-ORC1+ BML-284 (10 μM) groups. Cell viability (a and b), apoptosis (c), migration (d), and invasion (e) were detected in H358 and H1435 cells from each group by CCK-8, TUNEL, and transwell assays. n = 3. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

apoptosis [29]. However, the role of ORC1 in LUAD has not been systematically studied.

In this study, bioinformatics analysis was performed based on GTEx and TCGA databases to analyze ORC1 expression in pan-cancer. It was revealed that ORC1 was upregulated in most human cancers, including LUAD, which is consistent with current reports [15–17]. To thoroughly explore the relationship between ORC1 and LUAD, RNA-seq data and clinicopathological data from TCGA-LUAD dataset were analyzed. It was found that ORC1 was overexpressed in LUAD tissues in comparison to normal tissues. Additionally, high ORC1 expression was correlative to poor outcome in LUAD. ROC curve analysis showed that ORC1 expression was of certain prognostic value for the evaluation of survival time of LUAD patients. Also, ORC1 expression was positively related to LUAD grading, suggesting that ORC1 is associated with LUAD progression. Currently, emerging evidence has demonstrated a correlation between tumor progression and immune infiltration in LUAD [19,30]. Herein, we also found a certain correlation between ORC1

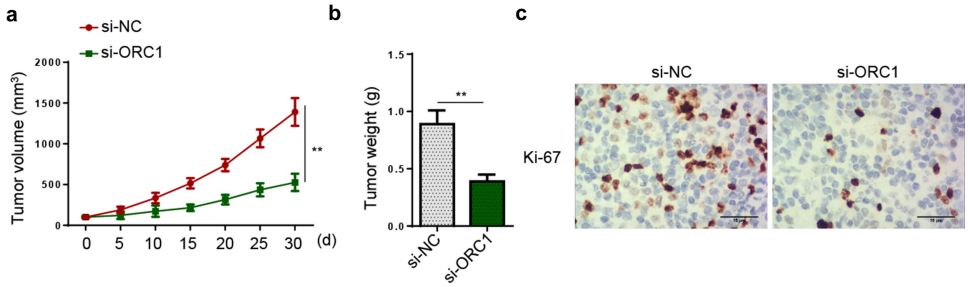

Figure 9. ORC1 facilitates LUAD tumor growth. (a) H358 cells transfected with si-NC or si-ORC1 were injected into BALB/c nude mice to construct xenograft tumor models. Tumor volume was measured every 5 days. (b) 30 days later, all mice were sacrificed to harvest tumors. The xenograft tumors were weighed. (c) Ki-67 staining of xenograft tumor. n = 3. $^{**}P < 0.01$.

expression and immune infiltration in LUAD. All these results insinuated that ORC1 might be oncogenic and of certain prognostic value in LUAD.

To analyze the specific function of ORC1 in LUAD, we also analyzed ORC1-related genes in LUAD. According to GO analysis performed based on these ORC1-coexpressed genes, it was found that the biological functions of ORC1 were mainly associated with inhibitory extracellular ligand-gated ion channel activity and GABA−gated chloride ion channel activity. Subsequently, *in vitro* and *in vivo* experiments were performed to further verify the upregulation of ORC1 in LUAD and further investigate its role and function in LUAD. RT-qPCR and western blotting assays showed that ORC1 was highly expressed in LUAD cell lines when compared to normal cell line. Next, we induced ORC1 silencing and overexpression in two LUAD cell lines with higher ORC1 expression. Functional experiments showed that ORC1 overexpression facilitated LUAD cell proliferation and metastasis but repressed LUAD cell apoptosis; however, ORC1 knockdown had the opposite effect. Also, animal experiments also affirmed the promoting role of ORC1 in LUAD tumor growth. The above results demonstrated that ORC1 may contribute to LUAD progression *in vitro* and in *vivo*. Hence, ORC1 might be a target to impede LUAD development.

To delve into the underlying mechanism of ORC1 in LUAD, GSEA analysis was performed based on TCGA-LUAD dataset. It was revealed that the Wnt signaling was positively correlated with ORC1 expression in LUAD. Recently, the significant role of Wnt signaling in LUAD pathogenesis has been convincingly substantiated [22]. For example, Destrin activates the Wnt/β-catenin signaling to promote LUAD cell proliferation and metastasis [31]. Besides, PHLDA3-mediated Wnt signaling activation may accelerate tumor growth and metastasis in LUAD [32]. Additionally, RFC3-induced inactivation of Wnt/β-catenin signaling represses LUAD progression by increasing cell apoptosis and suppressing cell proliferation and metastasis [33]. Considering the crucial role of Wnt signaling in LUAD progression and metastasis, it was hypothesized that the Wnt signaling was a vital pathway by which ORC1 exerted its regulatory effects in LUAD. Herein, BML-284, a Wnt activator, partially reversed the suppressive effects of ORC1 silencing on Wnt activation in LUAD cells, indicating ORC1 could confer the activation of Wnt signaling in LUAD cells. Further, rescue assays demonstrated that BML-284-induced Wnt activation abated the suppression on proliferative and metastatic capacities as well as the increase in the apoptotic rate of LUAD cells resulting from ORC1 knockdown. Taken together, ORC1 aggravated malignant behaviors in LUAD cells via targeting the Wnt signaling.

Conclusion

In this work, we illustrated that ORC1 could accelerate LUAD progression through activating the Wnt signaling, indicating ORC1 could be a latent biomarker and target in LUAD treatment. Our results help to deepen the understanding of molecular mechanisms involved in LUAD tumorigenesis. However, the lack of human clinicopathological analysis is still a limitation of our study. In the future, the correlation between ORC1 and

clinicopathological features of LUAD patients will be further investigated to verify our findings.

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