Identification of endonuclease domain-containing 1 as a novel tumor suppressor in prostate cancer

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Abstract

Background: Endonuclease domain containing 1 (ENDOD1) is implicated in tumorigenesis and aggressiveness of multiple tumors. In this study, we aimed to investigate the role of ENDOD1 in prostate cancer (PCa).

Methods: Immunohistochemistry were performed in 30 cases of benign prostatic hyperplasia (BPH) and 50 cases of PCa to identify its association with clinicopathological characteristics. Real-time PCR and western blot were used to detect ENDOD1 mRNA and protein expression in normal prostatic epithelial and PCa cell lines. MTT assays were employed to determine the effect of cell proliferation. Flow cytometry was used to explore the cell cycle distribution and apoptotic effects. Transwell migration and invasion assays were done to evaluate changes in the ability of cell migration and invasion.

Results: Immunoreactivity scores of ENDOD1 showed no statistical difference between BPH and low-grade PCa, whereas lower immunostaining scores were observed in high-grade compared with low-grade PCa. Real-time PCR data indicated that ENDOD1 mRNA expression was markedly increased in LNCaP and 22Rv1 cells and decreased in PC3 and DU145 cells compared to the normal epithelial cells RWPE1. Western blot showed that androgen-sensitive LNCaP cells had the highest protein expression level of ENDOD1, whereas castration-resistant PCa cell lines PC3 and DU145 had significantly lower protein levels. Meanwhile, overexpression of ENDOD1 suppressed cell proliferation, induced G0/G1 cell cycle arrest and inhibited cell migration and invasion. Conversely, siRNA-mediated silencing of ENDOD1 promoted cell proliferation, migration and invasion. No apoptotic effects occurred upon manipulation of ENDOD1 expression.

Conclusion: Our results indicate that ENDOD1 is a novel tumor suppressor in PCa, which may be employed as a new drug target of preventing progression to metastatic castration-resistant prostate cancer.

Keywords: Prostate cancer, Endonuclease domain-containing 1, Tumor suppressor

Background

Prostate cancer (PCa) is the most prevalent malignant cancer and the second leading cause of cancer-related death in men in the developed countries [1]. Although the incidence of PCa in China was obviously lower than that in western developed countries, it has kept increasing greatly for the past decades [2]. Treatment approaches vary according to individual situation. The localized PCa can be cured by the radical prostatectomy or definitive radiation therapy [3, 4]. Androgen deprivation therapy (ADT) is the mainstay treatment for the recurrent and advanced PCa and initially bring great treatment benefits, but most cases eventually progress into the lethal stage which is insensitive to ADT, namely castration resistant prostate cancer (CRPC) [5, 6]. Meanwhile, metastasis often occurred in CRPC patients and is the main cause of death [7, 8]. Although studies identify many biomolecules which help uncover the underlying mechanisms of PCa progression to CRPC and metastasis, the translational
applications in clinics are far from satisfaction [9, 10]. Therefore, exploring the novel biomarkers which play critical role in CRPC progression and metastasis may aid in the clinical decision-making to block disease progression, prolong patient survival and improve their quality of life.

Endonuclease domain containing 1 (ENDOD1) is a member of nucleases, which hydrolyze phosphodiester linkage in nucleic acids. It has been reported that nucleases participate in mutation avoidance, DNA repair and programmed cell death [11–13]. In addition, ENDOD1 belongs to the subgroup of non-sugar specific nuclease which hydrolyze both DNA and RNA without any apparent base preference [14]. Aberrant expression of ENDOD1 has been observed in several types of tumors. In soft tissue tumors, decreased ENDOD1 correlated with local aggressiveness [15]. Similarly, downregulation of ENDOD1 was revealed in colorectal cancer compared to the normal mucosa and proposed to be involved in epithelial tumorogenesis [16]. Moreover, quantitative real-time polymerase-chain-reaction (RT-PCR) analysis revealed a decreased mRNA expression of ENDOD1 in high-grade and lymphnode-metastatic PCa [17]. Nevertheless, the biological function of ENDOD1 in PCa remains to be clarified.

In this study, we determined the protein expression of ENDOD1 in PCa tissues by immunohistochemistry (IHC) and explored its correlations with clinicopathological parameters. We for the first time investigated the role of ENODD1 in cell proliferation, apoptosis, migration and invasion in PCa cells. Our findings showed that ENDOD1 protein expression exhibited a significant down-regulation in high-grade PCa tissues. In vitro studies indicated ENDOD1 inhibited cell proliferation, migration and invasion in PCa cells.

Methods

Human prostatic tissues and immunohistochemistry (IHC)

We examined a total of 30 BPH samples and 50 PCa samples in this study. The diagnosis was confirmed histologically after surgery. The BPH tissues and PCa tissues were respectively from patients treated by transurethral resection of prostate and laparoscopic radical prostatectomy in the Third Affiliated Hospital of Sun Yat-sen University during 1st January 2012 and 31st December 2015. IHC was performed as described previously [18]. Briefly, tissues were deparaffinized and then antigen retrieval was done in 10 mmol/L citrate buffer (pH 6.0) for 15 min at 100 °C. After blocking endogenous peroxidase with methanol containing 0.3% hydrogen peroxide (H2O2) for 15 mins, tissue sections were incubated overnight at 4 °C using a rabbit polyclonal ENDOD1 antibody (Abcam, ab121293, 1:500). All immunostained sections were reviewed by two pathologists independently who were blinded to the patients’ clinicopathological information. The analysis of immunostaining intensity was acquired by multiplying the level of staining intensity (negative = 0, weak = 1, moderate = 2, strong = 3) and percentage of positively stained cells (0% = 0, <10% = 1, 11–50% = 2, 51–80% = 3, >80% = 4), as described previously [19]. This study was approved by our Institutional Ethics Committee and all patients had signed the informed consents for using their surgically-resected tissues and related information.

Cell lines and cell culture

Human prostatic cancer cell lines DU145 (HTB-81™), PC3 (CRL-1435™), LNCaP (CRL-1740™) and 22Rv1 (CRL-2505™) and the non-malignant human prostatic normal epithelial cell RWPE-1 (CRL-11609™) were obtained from the American Type Culture Collection (ATCC) and routinely cultured in RPMI1640 medium (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS) in a humidified 37 °C incubator containing 5% CO2, according to the ATCC instructions.

RNA isolation and qPCR

Total RNA was extracted from RWPE-1, DU145, PC3, LNCaP and 22Rv1 using Trizol reagent (Invitrogen Life Technologies, CA, USA). cDNA was synthesized using 2 μg total RNA and SYBR® premix Ex Taq II (Takara, Japan). qPCR was performed on the ABI Prism 7500 fast Sequence Detection System (Applied Biosystem). The 2-ΔΔCt method was performed to analyze relative quantities for the level of gene expression. The following primers were used: GAPDH forward 5′-TCCTCTG ACTTCAACAGGCACACC-3′ and reverse 5′-TCTCTC TTCCTCTTGTGCTCCTTG-3′; ENDOD1 forward 5′- GACCGCATCCCCGTGTA-3′ and reverse 5′-AATCG CCTCCTCAAGGT-3′.

Transfection with expression vectors and small interfering RNA (siRNA)

The ENDOD1-expressing construct pCMV-N-Flag-ENDOD1 was performed as previously reported [20]. Genomic cDNA from PC3 cells was used as templates to amplify the upper and lower genes with the sites of EcoR I and Xho I restriction enzymes. Taq enzyme was used to obtain amplifications. After digested by the Xho I and EcoR I restriction enzymes, ENDOD1 and pCMV-N-Flag plasmid were extracted and purified using Gel Extraction Kit (Omega biotech) according to the manufacturer’s instructions. Then products were mixed, connected with DNA Ligation Kit (Takara) and transformed into DH5α peptide (Takara). After coated the lysogeny broth solid medium with kanamycin 100 μg/ml for 16 h, single colonies were chosen randomly. Colonies were inoculated in the
lysogeny broth medium containing with kanamycin 100 μg/ml overnight at 37 °C, 150 r/min and then plasmid DNA was extracted according to instructions of Endo-Free Plasmid Midi Kit (Omega biotech, China). The plasmids were sequenced before transfection (BGI, China). Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were also transfected with siRNA specific to ENDOD1 (5′-CAGAUUACCUGAUUCUGATT-3′) (GenePharma, Shanghai, China) using Lipofectamine 2000 (Invitrogen), following the manufacturer’s instructions. The concentration of siRNA used was 100 nM. After 6 h of transfection, the complete culture media was added. The assays were done 48 h post-transfection.

**Cell growth assay**
The DU145 with overexpression and negative control were cultured in 96-well culture plates at the number of 3000 cells per well, in RPMI-1640 medium containing 10% FBS. LNCaP transfected with siRNA and si-NC were cultured at a cell density of 5000 cells/well under the same conditions as DU145. The growth of the cells was analyzed by MTT assay (KeyGEN BioTECH, Nanjing, China). Optical density (OD), which was detected by an Automatic microplatereader (TEAN, Swiss) at a 490 nm wave length. The rate of cell proliferation was calculated. Three independent experiments were performed over multiple days.

**Flow cytometry (FCM)**
Cell cycle and apoptosis studies were performed using FCM on DU145 transfected with ENDOD1 overexpression and the control plasmids, LNCaP transfected with siRNA and si-NC, respectively. Cells were seeded at a concentration of 3 × 10^6 cells/mL. For cell cycle analysis, the cells were dispered in 500 μL PBS (1%, pH 7) with PI (50 μg/mL) and RNase A (100 μg/mL) and kept in dark for 15 min before FCM analysis. For apoptosis study, cells were treated with PI (50 μg/mL), annexin V-FITC conjugate (5 μg/mL)(KeyGEN BioTECH, Nanjing, China) and dispersed in PBS (1%, pH 7). Each analysis was performed at least thrice (n = 3) and a count of a minimum of 10,000 events was taken for each analysis. Data are represented as mean ± S.D.

**In vitro migration and invasion assays**
The migration and invasion activity of DU145 cells with overexpression ENDOD1 and LNCaP cells with siRNA were assessed using transwell migration assays and matrigel invasion assays as previously described [21]. Cells were seeded into the upper chamber of the transwell in 24-well plates (membrane pore size, 8 μm; Corning Incorporated; Corning, NY, USA) with Matrigel (BD Pharmingen), and 500 μl RPMI-1640 medium with 15% FBS was added into the lower chamber. After 24 h, the bottoms of the inserts were fixed in paraformaldehyde solution for 10 mins and stained with 0.1% crystal violet staining solution. The cells invading into the bottom-lower surfaces of inserts were measured by using microscope at ×100. Results were repeated in triplicate.

**Western blot analysis**
The western blot was performed as previously reported [22]. Cells were lysed by using RIPA buffer containing 1% PMSF. Protein concentration was quantified with the BCA Protein Kit (KeyGEN BioTECH, Nanjing, China) and equal amounts of proteins were loaded and separated by 12% SDS-PAGE, then transferred a to a PVDF membrane (Millpore, USA) and probed with primary antibody against ENDOD1 (Abcam, ab121293,1:2000) using antibodies against ENDOD1 (Abcam, ab121293, 1:2000). All the levels of protein were normalized to GAPDH (Abcam, ab9485,1:2500).

**Statistical analysis**
Data are expressed as the means ± SD. Data significance was analyzed using Student’s t-test, Mann–Whitney U-test and Chi-square χ² tests. A value of P < 0.05 was considered to indicate a statistically significant difference. All analyses were performed with SPSS version 19.0 (SPSS Inc., Chicago, IL, USA).

**Results**
**ENDOD1 immunostaining and correlation with clinicopathological factors of PCa**
We detected ENDOD1 protein expression in 30 BPH patients and 50 PCa patients by IHC. The mean ages of BPH patients and PCa patients were 54.2 (45–84) years and 68.3 (50–82) years, respectively. The average PSA value was 9.4 (2.1–25.6) ng/mL in BPH patients and 24.2 (3.8–175.5) ng/mL in PCa patients. The basic clinical characteristics of PCa patients are summarized in Table 1. No association was found between ENDOD1 expression and age (P = 0.749), preoperative PSA (P = 0.196), clinical T stage (P = 0.443), biopsy Gleason score (P = 0.191), pathological T stage (P = 0.292), seminal vesicle invasion (P = 0.139), lymphnode involvement (P = 0.064). While a significant difference was revealed between ENDOD1 expression and postoperative Gleason score (P = 0.011) and surgical margin status (P = 0.024). Representative images of immunostaining in BPH tissues and PCa tissues are shown in Fig. 1a-f. Immunoreactivity score analysis demonstrated that there was no difference in ENDOD1 expression between BPH samples and PCa tissues with low Gleason score (Gleason score < 7) (Fig. 1g).
However, PCa tissues with high Gleason score (Gleason score ≥ 7) displayed significantly lower ENDOD1 expression than that with low Gleason score and BPH (Fig. 1g). These findings suggested that ENDOD1 down regulation might play a role in the progression of PCa.

ENDOD1 expression in PCa cell lines
In order to investigate the expression of ENDOD1 in PCa cells, Real-time PCR analysis of ENDOD1 mRNA expression was performed in a panel of normal prostatic epithelial and PCa cell lines (22Rv1, LNCaP, PC3 and DU145). The data indicated that the level of ENDOD1 expression was markedly increased in LNCaP and 22Rv1 cells lines (mean ± SD, n = 3, P < 0.05) and decreased in PC3 and DU145 (mean ± SD, n = 3, P < 0.05) compared to the normal epithelial cell RWPE1 (Fig. 2a). Western blot showed that androgen-sensitive LNCaP cells had the highest protein expression level of ENDOD1, whereas castration resistant PCa cell lines PC3 and DU145 had significantly lower levels of ENDOD1.

Table 1 Patients characteristics and ENDOD1 expression of 50 prostate cancer patients

|                         | Total no. cases (%) | ENDOD1 expression | P-value |
|-------------------------|---------------------|-------------------|---------|
|                         | Low or none no. cases (%) | High no. cases (%) |         |
| All cases               | 50 (100)            | 22 (100)          | 28 (100) |
| Age                     |                     |                   |         |
| <69                     | 24 (48)             | 10 (45.5)         | 14 (50)  |
| ≥69                     | 26 (52)             | 12 (54.5)         | 14 (50)  |
| PSA (ng/ml)             |                     |                   |         |
| <10                     | 25 (50)             | 8 (36.4)          | 17 (60.7) |
| 10–20                   | 12 (24)             | 6 (27.2)          | 6 (21.4)  |
| >20                     | 13 (26)             | 8 (36.4)          | 5 (17.9)  |
| Clinical T stage        |                     |                   |         |
| cT1                     | 37(74)              | 15(68.2)          | 22(78.6) |
| cT2                     | 12(24)              | 6(27.3)           | 6(21.4)  |
| cT3                     | 1(2)                | 1(4.5)            | 0(0)     |
| Biopsy Gleason score    |                     |                   |         |
| ≤6                      | 25(50)              | 9(40.9)           | 16(57.1) |
| 7                       | 13(24)              | 5(22.7)           | 8(28.6)  |
| 8–10                    | 12(26)              | 8(36.4)           | 4(14.3)  |
| Pathological T stage    |                     |                   |         |
| pT2                     | 33(66)              | 12(54.5)          | 21(75)   |
| pT3a                    | 4(8)                | 2(9.1)            | 2(7.1)   |
| pT3b                    | 13(26)              | 8(36.4)           | 5(17.9)  |
| Post-op Gleason score   |                     |                   |         |
| ≤6                      | 22(44)              | 6(27.3)           | 16(57.1) |
| 7                       | 10(20)              | 3(13.6)           | 7(25)    |
| 8–10                    | 18(36)              | 13(59.1)          | 5(17.9)  |
| Seminal vesicle invasion|                     |                   |         |
| Yes                     | 13(26)              | 8(36.4)           | 5(17.9)  |
| No                      | 37(74)              | 14(63.6)          | 23(82.1) |
| Lymphnode involvement   |                     |                   |         |
| Yes                     | 10(20)              | 7(31.8)           | 3(10.7)  |
| No                      | 40(80)              | 15(68.2)          | 25(89.3) |
| Surgical margin status  |                     |                   |         |
| Yes                     | 9(18)               | 7(31.8)           | 2(7.2)   |
| No                      | 41(82)              | 15(68.2)          | 26(92.9) |
However, the protein level is not proportional to the mRNA level in 22RV1 (Fig. 2b and c). These results indicated that ENDOD1 may be involved in the progression from androgen dependence (AD) to androgen independence (AI). For further investigation on the impact of ENDOD1 in tumor biology of PCa, we selected LNCaP and DU145 cells as study models.

ENDOD1 suppresses the proliferation of PCa cells
To explore the effect of ENDOD1 on proliferation of PCa cells, eukaryotic overexpression plasmid pCMV-N-Flag-ENDOD1 and specific small siRNA was transfected into DU145 and LNCaP cells respectively. The expression of ENDOD1 was confirmed by western blot (Fig. 3a and b). We next investigated the cell proliferation rate of these cells by MTT assay. After transfection with ENDOD1, DU145 cells showed significantly decreased OD values in 96 h post-transfection, suggesting an inhibitory effect of ENDOD1 on proliferation of DU145 ($P < 0.05$, Fig. 3c). Conversely, LNCaP cells proliferated remarkably faster after siRNA-mediated down regulation of ENDOD1 ($P < 0.05$, Fig. 3d). Therefore, ENDOD1 suppresses cell proliferation in PCa cells.

Effects of ENDOD1 on cell cycle and apoptosis
To identify changes in cell cycle distribution, we performed FCM after manipulation of ENDOD1 expression. Overexpression of ENDOD1 in DU145 cells led to
notable increases in G0/G1 phase arrest, with a corresponding decrease in the percentage of cells in the G2/M and S phases, compared with control group (Fig. 4a and b, \( P < 0.01 \)). LNCaP cells transfected with siRNA reversed the effect, with a remarkable increase in the percentage of cells in the G2/M phase (Fig. 4c and d, \( P < 0.01 \)). Moreover, we measured cell apoptotic rate to determine whether ENDOD1 promotes apoptosis. As shown in Fig. 4e and f, the percentage of apoptotic cells were 3.28% vs 1.02% for DU145 cells with ENDOD1 expression and the control, respectively and were 5.53% vs 5.66% for LNCaP cells with siRNA and si-NC, respectively, showing no statistical difference. Collectively, these data suggest that ENDOD1 inhibits cell cycle progression at the G0/G1 arrest and has no effect on cell apoptosis.

**ENDOD1 inhibits migration and invasion in PCa cells**

As for the role of ENDOD1 in migration and invasion of PCa cells, we performed transwell migration and invasion assays. ENDOD1 overexpression significantly suppressed cell migration and invasion in DU145 (Fig. 5a and c) whereas knockdown of ENDOD1 by siRNA promoted cell migration and invasion in LNCaP cells (Fig. 5b and d). Taken together, there is an obvious negative regulation of migration and invasion by ENDOD1 expression in PCa cells.

**Discussion**

Metastatic castration-resistant prostate cancer (mCRPC) is the lethal and incurable stage of PCa. Currently, the overall survival of patients with mCRPC is about 40.7 months regardless of multidisciplinary treatment modality [23]. Researches on the mechanisms whereby PCa progresses to mCRPC identify large numbers of oncotargets, but few translate into clinical implication [6, 24]. Therefore, any efforts on discovering biomarkers that involve in PCa progression are worthwhile and may bring breakthrough in target-directed treatments in PCa.

It is reported that endonuclease domain-containing 1 (ENDOD1) is aberrantly deregulated in several tumors. Gaedcke J et al. proposed that ENDOD1 involved in epithelial tumorigenesis of colorectal cancer and could be used as a novel tumor marker [16]. Our results didn't showed differences in ENDOD1 expression in tissues between BPH group and low-grade PCa group whereas significant downregulation was observed in high-grade PCa group, suggesting ENDOD1 may exert aggressiveness-promoting function in PCa. Cunha IW et al. revealed inverse correlation between ENDOD1 expression and local aggressiveness in soft tissue tumors and reported that ENDOD1 may play a role in tumor biology [15]. Consistently, we demonstrated that the ENDOD1 expression in PCa tissues was negatively correlated with Gleason score which is widely considered as a marker of aggressiveness. These results indicate a negative role of ENDOD1 in tumor progression.

In PCa cell lines, ENDOD1 expression in mCRPC cells were significantly decreased compared with androgen-sensitive LNCaP cells, suggesting ENDOD1 may promote PCa progression to CRPC. Marques RB et al. demonstrated that ENDOD1 is regulated genes of androgen receptor (AR) in hormonal therapy-resistant PCa cells [17]. Since AR plays critical role in promoting PCa tumor growth and progression [25], ENDOD1 is likely to be the mediator of AR signaling in the progression to mCRPC and could possibly be used as markers in predicting the course of disease. It should be noted that the protein level is not proportional to the mRNA of ENDOD1 in 22RV1 cells. This
could be caused by a multitude of post-transcriptional mechanisms such as mRNA and protein degradation [26], but the specific mechanisms need further investigation.

Moreover, we found that ENDOD1 inhibited cell proliferation, migration and invasion. Of literatures, ENDOD1 is a member of the DNA/RNA sugar non-specific nucleases [14]. Nucleases are implicated in mutation avoidance, DNA repair and programmed cell death via hydrolyzing phosphodiester linkage in nucleic acids [11–13]. Aberrant alteration of mutation, DNA repair and programmed cell death often occurred in PCa and were considered to associate with cancer predispositions and progression [27–29]. Therefore, there’s probability that ENDOD1 functions as tumor suppressor through preventing aberrant alteration of mutation, DNA repair and programmed cell death. However, it needs further investigation to elucidate the specific mechanisms.

There are some limitations in our study. Firstly, the number of PCa patients is relatively small, but it is sufficient to guarantee the validity of statistical significance. Secondly, follow-up data for the PCa patients hasn’t been analyzed to explore the prognostic value of ENDOD1 in PCa. Thirdly, the specific mechanisms whereby ENDOD1 exert inhibitory activity remain elusive. Nevertheless, our study identified ENDOD1 is a novel tumor suppressor, which may add our knowledge about PCa progression.

**Conclusion**

In this study, we have identified ENDOD1 as a novel tumor suppressor in PCa. ENDOD1 may be employed as drug target to prevent disease progression to mCRPC.

**Abbreviations**

AD: androgen dependence; ADT: androgen deprivation therapy; AI: androgen independence; ATCC: American Type Culture Collection; BPH: benign prostatic hyperplasia; CRPC: castration resistant prostate cancer; ENDOD1: endonuclease domain containing 1; FBS: fetal bovine serum; FCM: flow cytometry; IHC: immunohistochemistry; mCRPC: metastatic castration-resistant prostate cancer; OD: optical density; PCa: prostate cancer; siRNA: small interfering RNA

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**Availability of data and materials**

The datasets during and/or analyzed during the current study are available from the corresponding author on reasonable requests.

**Authors’ contributions**

JGQ performed the operations, collected the tissue samples, participated in the results analysis and drafted the manuscript. SBP carried out the cells culture, performed mRNA and protein studies. JST helped perform the operations, and the analysis with constructive discussions. CH carried out IHC studies and helped to analyze the data. WTH have made substantial contributions to acquisition of samples and data and involved in drafting the manuscript. YHM has been involved in drafting the manuscript and revising...
the manuscript critically. WHQ contributed to the data analyses and manuscript preparation. DJW conceived of the study, and participated in its design and coordination. KL participated in the design of the study and its coordination and has been involved in drafting the manuscript, revising it critically for important intellectual content. All authors have read and approved the final version of this manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
The study was approved by the institutional review board and the ethics committee of the Third Affiliated Hospital of Sun Yat-sen University. Written informed consent was obtained from all patients.

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Fig. 4 Effects of ENDOD1 on cell cycle and apoptosis. a-b Cell cycle distribution without or with overexpression of ENDOD1 in DU145 cells. Overexpression of ENDOD1 led to notable increases in G0/G1 phase arrest, with a corresponding decrease in the percentage of cells in the G2/M and S phases. c-d Cell cycle distribution after transfecting siRNA or si-NC of ENDOD1 in LNCaP cells. siRNA-induced downregulation of ENDOD1 led to remarkable increase in the percentage of cells in the G2/M phase. e-f FITC-Annexin V test demonstrated no changes in apoptosis between the control group and the group of ENDOD1 overexpression (e) or siRNA (f). Error bars represent the means of three independent experiments. *P <0.05.
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