

# Obesity accelerates murine gastric cancer growth by modulating the Sirt1/YAP pathway

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**Abstract.** A previous study from our group using an *in vivo* model demonstrated that diet induced-obesity increases the risk of gastric cancer and may prompt its growth. However, the molecular mechanisms underlying this association remain unclear and require further investigation. The aim of the present study was to investigate the potential molecular mechanisms through which obesity affects gastric cancer growth. In a subcutaneous mouse model, tumors were significantly larger in obese mice compared with non-obese and lean mice. In addition, markedly increased levels of Sirt1 and YAP protein were observed in the nucleus of cells from subcutaneous tumors from obese mice compared with those from lean mice. Murine forestomach carcinoma (MFC) cells treated with 5% sera from obese mice exhibited significantly increased expression of Sirt1 and YAP compared with MFC cells treated with sera from lean mice. In addition, a positive correlation was observed between Sirt1 expression and YAP expression, and between Sirt1 expression and serum visfatin levels in mice. These results suggested that diet-induced obesity could promote murine gastric cancer growth by modulating the Sirt1/YAP signaling pathway.

## Introduction

Gastric cancer is the fifth most common form of malignant tumor and the third leading cause of cancer-associated mortality worldwide (1). Due to the aggressiveness of gastric cancer biology and the limited effectiveness of current therapeutic modalities with advanced disorders, further studies are required in order to understand the underlying molecular mechanisms of gastric cancer growth and identify the potential of novel targets for therapeutic intervention.

Obesity, a worldwide epidemic (2), is associated with an increased risk of numerous types of cancer including colorectal, postmenopausal breast, prostate and renal cancer (3), and esophageal and gastric adenocarcinoma (4). Obesity may promote the growth of gastric cancer through complex molecular mechanisms, which require further investigation. The molecular mechanisms by which obesity affects cancer growth are currently being investigated; adipokine, hormonal, inflammatory and immunological changes may contribute in part to altered tumor biology (5). The most abundant adipokines derived from adipose tissue have been implicated as mediators of the effects of obesity on cancer development (6). The level of one such adipokine, visfatin, increases in obese individuals and contributes to a general pro-inflammatory state in the peripheral organs (7). This may prove to be an important mechanistic link in the network of factors affecting obesity-associated tumor growth. Visfatin acts as a NAD biosynthetic enzyme similar to nicotinamide phosphoribosyl transferase (Nampt) (8), functioning as an enzyme involved in the NAD<sup>+</sup> salvage pathway, which was demonstrated to be upregulated in numerous types of human malignant tumors (9), including gastric cancer (10).

Silent mating-type information regulation 2 homolog 1 (Sirt1), originally identified as a longevity gene, is induced by caloric restriction and regulates various cellular functions, including DNA repair, metabolism and cell survival under genotoxic and oxidative stress (11). Sirt1 functions as a NAD<sup>+</sup>-dependent histone deacetylase, and deacetylates a number of key cell cycle proteins and apoptosis regulatory molecules, including tumor protein p53 (12,13). The role of Sirt1 in cancers has been studied previously; however, whether Sirt1 serves a role as a tumor suppressor or tumor promoter remains unclear, since it seems to depend on the cellular context, its targets in specific signaling pathways or the specific type of cancer (11). Previous reports have demonstrated that Sirt1 is involved in carcinogenesis and enhances the growth, metastasis and chemotherapy resistance of a number of cancers through its anti-apoptotic activity, including colon (14), breast (15) and gastric cancer (16). Elevated Sirt1 deacetylates activated p53 (17) and this allows cells with damaged DNA to proliferate, promoting tumor development (18).

Yes-associated protein (YAP), a transcriptional co-activator that acts downstream of the Hippo signaling

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pathway, regulates multiple cellular processes and is associated with tumor growth and development (19). The YAP gene locus is amplified in human malignancies, including glioma, medulloblastoma (20), oral squamous cell carcinoma and hepatocellular carcinoma (HCC) (21). Consistently, upregulated YAP expression and nuclear localization have been observed in multiple types of human malignant tumors, including liver, colon, ovarian, lung and prostate cancer (22). Furthermore, YAP has been associated with tumor development and the prognosis of patients with cancer (21). YAP shuttles between the cytoplasm and the nucleus, where it induces the expression of pro-proliferative and anti-apoptotic genes via interactions with transcription factors, particularly TEA domain (TEAD) family members (23). When the upstream Hippo kinase receives an extracellular growth inhibition signal, YAP is phosphorylated and inactivated, which results in the inhibition of transcriptional activity through the cytoplasmic retention of YAP and subsequent ubiquitin-mediated proteasomal degradation, therefore gene expression is downregulated (23). By contrast, when the kinase receives a growth promoting signal, hypo-phosphorylated YAP translocates into the nucleus and induces target gene expression (24) to regulate tissue homeostasis, organ size, regeneration and tumorigenesis.

A Sirt1-YAP signaling pathway in which YAP is regulated by Sirt1-mediated deacetylation has been identified in cancer cells; a cycle of acetylation/deacetylation of nuclear YAP exists downstream of the Hippo signaling pathway (25). The deacetylation of YAP2 by Sirt1 promotes the YAP2/TEAD4 association, resulting in YAP2/TEAD4 transcriptional activation and cell growth in HCC cells, and Sirt1 promotes cisplatin (CDDP)-induced YAP2 nuclear accumulation and inhibits CDDP-induced apoptosis (26,27).

Until now, to the best of our knowledge, few studies have been published regarding the mechanisms by which obesity affects gastric cancer growth. Our group previously developed a model of murine gastric cancer using C57BL/6j high fat dietary obese mice and flank-implanted murine gastric cancer cells. The results demonstrated that diet-induced obese mice exhibited metabolic changes and larger subcutaneous tumors than lean mice (28). In addition, histological analyses demonstrated that obesity not only enhanced cellular growth, but also reduced cellular apoptosis (28). The aim of the present study was to use this *in vivo* mouse model to investigate the molecular mechanisms underlying the association of obesity with gastric cancer growth.

## Materials and methods

**Cell culture.** The gastric cancer cell line used in this study, murine forestomach carcinoma cell line (MFC), was purchased from The Cell Bank Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) (29). MFC cells were maintained in RPMI-1640 medium (Cellgro; Corning Incorporated, Corning, NY, USA) containing 10% fetal bovine serum (Valley Biomedical Products & Services, Inc., Winchester, VA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were maintained in a 37°C humidified incubator with 5% CO<sub>2</sub>.

**Diet induced obesity model and *in vivo* gastric cancer model.** Three- to five-week-old male C57BL/6j mice (n=36) were obtained from Shanghai Laboratory Animal Center (Chinese Academy of Sciences, Shanghai, China) and bred under standard conditions, controlled at a temperature of 20-25°C and 40-50% humidity with a 12 h light/dark cycle, and the body weight of these animals ranged between 15-18 g. Mice were randomly divided into two groups, then were weaned onto a high-fat diet (35.5% fat, 36.3% carbohydrate, 20.0% protein, n=24) and normal diet (5.4% fat, 51.0% carbohydrate, 22.9% protein, n=12) (30), respectively for 12 weeks, and then mice were divided into three groups (lean, obese and non-obese) as previously described (31). The mice in different groups were maintained on their previous diet until the end of the experiment. The mice were allowed access to their specific diet and water *ad libitum*.

To detect the impact of obesity on tumor growth, each of 12 obese, 12 lean and 12 non-obese mice was injected subcutaneously with 2.0x10<sup>6</sup> MFC cells into the right flank and monitored daily to check for the presence of palpable tumors. Then all mice were maintained on normal or high fat diet for another 2 weeks. Tumor growth was observed in 100% of the obese animals, in 75% (9/12) of the non-obese mice and in 83.3% (10/12) of the lean mice. In all experiments, body weight and food intake were checked twice per week. At the end of experiment, following overnight fasting, the animals were sacrificed, and tumor tissues were extracted, immediately frozen in liquid nitrogen, and stored at -80°C until RNA and protein extraction. The tumor volumes measured range between 8 and 150 mm<sup>3</sup>.

All experiments were approved by the Xi'an Jiaotong University Institutional Animal Care and Use Committee following 'Principles of laboratory animal care' (NIH publication no. 85-23, revised 1985). All surgery was performed under sodium pentobarbital anesthesia and all efforts were made to minimize suffering.

***In vitro* study of the effect of obesity on Sirt1/YAP.** Following overnight fasting and anesthesia, the blood of these mice was obtained via cardiac puncturing. Sera samples were separated by centrifugation for 10 min at 2,000 x g and 4°C and stored at -80°C until measurements were performed. Biological behaviors of MFC cells induced by sera of mice were analyzed as previously reported (31). The expression of Sirt1 and YAP was examined in cultured cells with exposure to RPMI-1640 or 5% sera of obese mice or lean animals to detect whether endocrine mechanism of obesity may be responsible for the growth of MFC cells.

**Immunohistochemical analysis.** Tumors were obtained from the *in vivo* xenograft model and fixed in 10% neutral buffered formalin for 24 h at room temperature, then were embedded in paraffin. The paraffin blocks were cut on a microtome into 5 mm-thick sections. The tumor sections were dewaxed and dehydrated with descending alcohol series (10 min for 100% alcohol, then 5 min for 95, 90 and 80% alcohol). Rehydration, antigen retrieval in citrate buffer, endogenous peroxidase activity was blocked for 10 min using 3.0% hydrogen peroxide at room temperature, then the sections were blocked with 10% goat plasma (cat. no. SAP-9100; ZSGB-BIO, Beijing, China)

Table I. List of antibodies and dilutions used in the present study.

Protein	Experiment	Final concentration	Catalog number	Supplier
Sirt1	WB	1:200	sc-15404	Santa Cruz Biotechnology, Inc., Dallas, TX, USA
	IHC	1:50	sc-15404	Santa Cruz Biotechnology, Inc.
YAP	WB	1:200	ab52771	Abcam, Cambridge, UK
	IHC	1:50	ab52771	Abcam
GAPDH	WB	1:500	sc-47724	Santa Cruz Biotechnology, Inc.

WB, western blotting; IHC, immunohistochemistry; Sirt1, silent mating-type information regulation 2 homolog 1; YAP, Yes-associated protein.

Table II. List of primer sequences.

Gene	Primer	5'→3' Sequences	PCR size (bp)
GAPDH	Forward	CGTAGACAAAATGGTGAAGG	296
	Reverse	GACTCCACGACATACTCAGC	
Sirt1	Forward	TTGTGAAGCTGTTCGTGGAG	412
	Reverse	GCGTGGAGGTTTTTCAGTA	
YAP	Forward	CCCTGATGATGTACCACTGCC	623
	Reverse	CCACTGTAAAGAAAGGGATCGG	

Sirt1, silent mating-type information regulation 2 homolog 1; YAP, Yes-associated protein; PCR, polymerase chain reaction.

for 30 min at room temperature, then separately incubated with the primary antibodies directed against Sirt1 and YAP (both rabbit anti-mouse) at 4°C overnight. The primary antibodies were detected using biotinylated secondary goat anti-rabbit antibodies (cat. no. SAP-9100; ZSGB-BIO, Beijing, China) for 30 min at room temperature following the manufacturer's recommendations. The staining of the sections was performed using the horseradish peroxidase-streptavidin conjugates for Sirt1 and YAP (SP method). The primary antibodies used and their experimental conditions are summarized in Table I. The scoring system for Sirt1 and YAP expression was performed as previously described (31). Briefly, staining intensity was expressed as four grades: 0, none; 1, weak; 2, moderate; and 3, strong. The percentage of positive MFC cells was expressed as: 0, <5%; 1, 6-25%; 2, 26-50%; 3, 51-75%; and 4, >75%. The staining intensity and average percentage of positive MFC cells were assayed for 10 independent high power fields (x400) with the help of Olympus microscope (type BHS, Japan). The total score was calculated by multiplying the staining intensity and the percentage of positive MFC cells. All histological analyses were carried out by three independent observers.

**RNA expression studies.** Total RNA was extracted from the target cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. To verify the stability of these mRNA, the products were separated on 0.8% agarose gel with 0.5 µg/ml ethidium bromide electrophoresis and visualized under ultraviolet light by a gel imaging analysis

system (vJS-2000; Pei & Qing Science & Technology, Inc., Shanghai, China). Expression of Sirt1 and YAP mRNA was quantified by reverse transcription-polymerase chain reaction (RT-PCR). RNA was reverse transcribed to complementary DNA using the High Capacity 1st Strand Synthesis kit (Takara Bio, Inc., Otsu, Japan). The PCR reaction was performed using an iCycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with the following thermocycling conditions: A pre-heating step at 95°C for 10 min followed by 30 repeats of 94.0°C for 30 sec and 55.0°C for 30 sec, then 72°C for 1 min. The products were separated on 2% agarose gel with 0.5 µg/ml ethidium bromide electrophoresis and visualized under ultraviolet light by a gel imaging analysis system (vJS-2000; Pei & Qing Science & Technology, Inc., Shanghai, China). The primer sequences were generated using National Center for Biotechnology Information Primer-BLAST and are presented in Table II. Transcript levels were normalized to GAPDH. For validation, each experiment was carried out in triplicate.

**Protein extraction and western blot analysis.** High quality nuclear protein for western blot analysis was extracted from the cultured cells by exposure to RPMI-1640 or 5% sera of obese mice or lean animals for 24 h with a Nuclear Protein Extraction kit (cat. no. P0028; Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. Protein concentration was measured using a BCA kit (Pierce; Thermo Fisher Scientific, Inc.). A total of 20 µg protein was loaded per lane, then were run on a 10% SDS-PAGE and were transferred to nitrocellulose

Table III. The weight of mice and subcutaneous tumors.

Parameters	Obese (n=12)	Non-obese (n=9)	Lean (n=10)
Body weight (g)	35.4±2.8 <sup>a</sup>	28.7±2.3 <sup>b</sup>	28.5±1.2
Tumor weight (mg)	134.2±17.3 <sup>a</sup>	83.4±15.3 <sup>b</sup>	77.2±14.9

<sup>a</sup>P<0.05 vs. lean and non-obese, <sup>b</sup>P>0.05 vs. lean.

membranes (EMD Millipore, Billerica, MA, USA) using a Bio-Rad Mini PROTEAN 3 System, according to the manufacturer's protocol. The nitrocellulose membranes were then blocked in TBST with 5% non-fat dry milk at 37°C for 2 h. Subsequently, the membranes were incubated with a 1:200 dilution of the primary antibodies for Sirt1 and YAP, and a 1:500 dilution of anti-GAPDH at 4°C overnight (Table I). Anti-rabbit (cat. no. ZB-2301; ZSGB-BIO, Beijing, China) or anti-mouse IgG (cat. no. sc-516102; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) antibodies with a 1:5,000 dilution were used as the secondary antibodies, and incubated with the membrane for 1 h at room temperature. The membranes were then developed with enhanced chemiluminescence (Pierce; Thermo Fisher Scientific, Inc.) by an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ, USA). For validation, each experiment was carried out in triplicate.

**Statistical analysis.** Values were expressed as the mean ± standard deviation. Statistical differences were estimated by one-way analysis of variance followed by a Dunnett's test or Spearman rank test using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

## Results

*Obese mice exhibit a larger tumor size and increased visfatin levels compared with non-obese and lean mice.* Mice were segregated by weight for further analysis as previously described (31). Obese, non-obese and lean mice were selected at the time of transplantation. The obese mice were significantly larger compared with the non-obese and lean mice in body weight (P<0.05; Table III). Tumor growth was observed in 100% of the obese animals, in 75% (9/12) of the non-obese mice and in 83.3% (10/12) of the lean mice. At the end of the experiment, the obese mice possessed significantly larger tumors compared with the non-obese and lean mice (both P<0.05; Table III), but there was no difference between lean mice and non-obese mice (P>0.05), and they exhibited a positive correlation with the final body weight of the mice (r=0.75; P<0.05; n=31).

Diet-induced obese mice have been demonstrated to exhibit metabolic changes, including insulin resistance, glucose intolerance, hyperglycemia and hyperinsulinemia, and altered adipokine levels (31). The serum visfatin levels from this previous study were as follows: Obese (n=12), 44.3±3.6 ng/ml (P<0.01 vs. non-obese and P<0.01 vs. lean);

non-obese (n=9), 38.9±2.7 ng/ml (P>0.05 vs. lean); lean (n=10), 39.6±3.4 ng/ml (31). This indicated that obesity may promote murine gastric cancer growth via endocrine mechanisms.

Since no significant differences were observed in tumor and body weight (P>0.05; Table III) or serum visfatin level and other metabolites (data not shown) between the non-obese and lean groups, further analyses were restricted to the obese and lean groups.

### *Expression of Sirt1 and YAP in tumors is positively correlated.*

The mechanisms by which obesity affects gastric cancer growth are complex. Obesity may promote gastric cancer growth via the pro-survival Sirt1/YAP signaling pathway (27). Therefore, the expression of Sirt1 and YAP was investigated in subcutaneous tumors by immunohistochemistry. Sirt1 and YAP localized primarily in the cell nucleus and their expression was significantly elevated in obese mice compared with the control (7.75±2.05 vs. 0.58±0.52; t=11.743; P<0.001 for Sirt1 and 9.08±1.68 vs. 1.25±0.62; t=15.176; P<0.001 for YAP; Figs. 1 and 2). The level of Sirt1 protein was positively correlated with that of YAP (r=0.94; P<0.001; n=22; Fig. 3A) in the tumors. In addition, the serum visfatin level was positively correlated with Sirt1 protein in the tumors (r=0.89; P<0.001; n=22; Fig. 3B).

### *Obesity promotes the growth of gastric cancer via the Sirt1/YAP pathway.*

The expression of Sirt1 and YAP was investigated in MFC cells cultured with 5% sera of obese or lean mice. Sirt1 and YAP were significantly upregulated at the mRNA and nuclear protein levels in cells treated with sera from obese mice compared with those treated with sera from lean mice and the control RPMI-1640 group without serum (both P<0.05; Fig. 4). This result suggests that obesity could promote MFC cell migration and proliferation, decrease MFC cellular apoptosis and accelerate cell cycle progression (31) by promoting the Sirt1/YAP signaling pathway.

## Discussion

In the present study, a gastric cancer *in vivo* model was used to investigate the association between obesity and gastric cancer growth. The subcutaneous tumor weight was significantly larger in obese C57BL/6j mice compared with non-obese and lean animals. No significant differences in body weight, tumor weight, the serum visfatin level and levels of other metabolites were identified between the non-obese and lean mice, indicating that obesity, but not the high-fat diet itself, promoted murine gastric cancer growth.

Obesity upregulates the expression level of pro-survival signals, which may shift the apoptotic balance of MFC cells towards survival (5). One target of this obesity-mediated effect is the Sirt1/YAP pro-survival pathway. In the present study, immunohistochemical analysis demonstrated that YAP expression in the MFC xenografts was elevated in obese mice compared with the lean mice, and its expression in cultured MFC cells was significantly increased at the mRNA and protein levels in obese mice compared with the lean mice. Although Sirt1 serves dual roles in the growth and progression

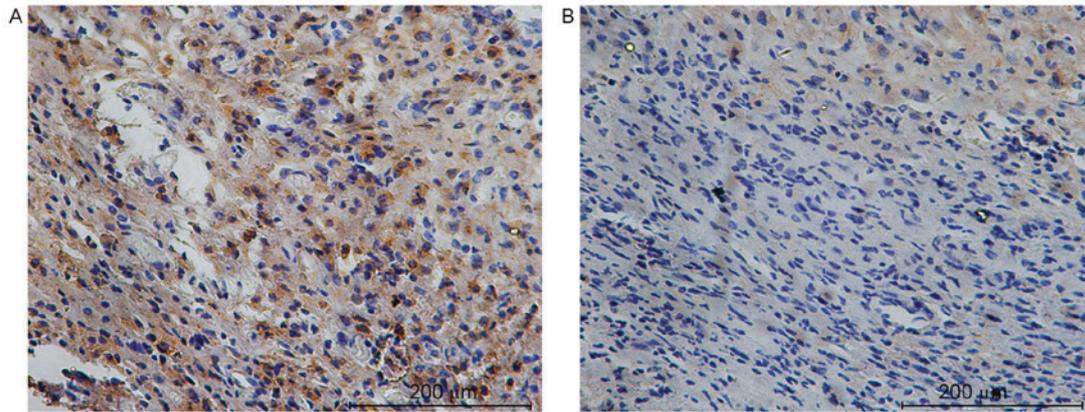


Figure 1. Sirt1 expression is elevated in subcutaneous tumors from obese mice compared with those from lean mice. The protein expression of Sirt1 was significantly elevated in the tumors from (A) obese mice (n=12) compared with those from (B) lean mice (n=10) ( $7.75 \pm 2.05$  vs.  $0.58 \pm 0.52$ ;  $t=11.743$ ;  $P<0.001$ ; magnification, x400). Sirt1, silent mating-type information regulation 2 homolog 1.

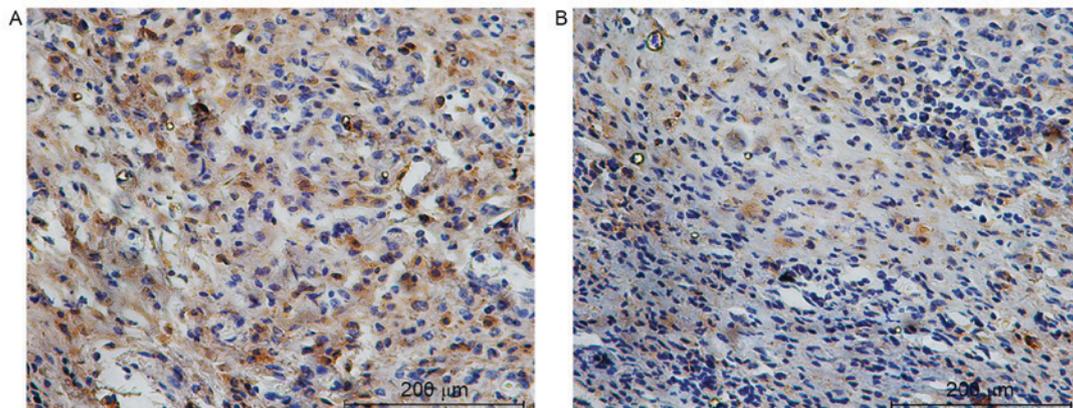


Figure 2. YAP protein expression is elevated in subcutaneous tumors from obese mice compared with those from lean mice. YAP protein expression was markedly increased in the subcutaneous tumors from (A) obese mice compared with those from (B) lean mice ( $9.08 \pm 1.68$  vs.  $1.25 \pm 0.62$ ;  $t=15.176$ ;  $P<0.001$ ; magnification, x400). YAP, Yes-associated protein.

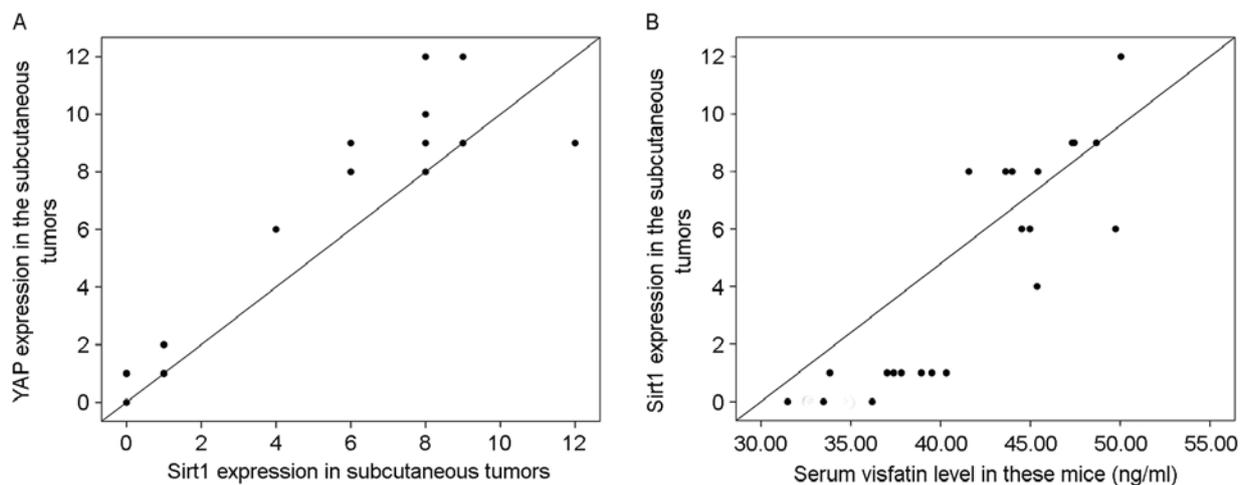


Figure 3. Sirt1 expression positively correlates with YAP expression and visfatin level in mouse tumors. (A) Correlation of Sirt1 expression with YAP expression ( $r=0.94$ ;  $P<0.001$ ) and (B) correlation of serum visfatin level with Sirt1 expression ( $r=0.89$ ;  $P<0.001$ ) in subcutaneous tumors from all mice. Sirt1, silent mating-type information regulation 2 homolog 1; YAP, Yes-associated protein.

of tumors (11), in the present study, Sirt1 was upregulated in tumors from obese mice, which may prompt the growth

of MFC cells *in vitro*. Therefore, obesity accelerates murine gastric cancer growth. However, how specific adipose tissues

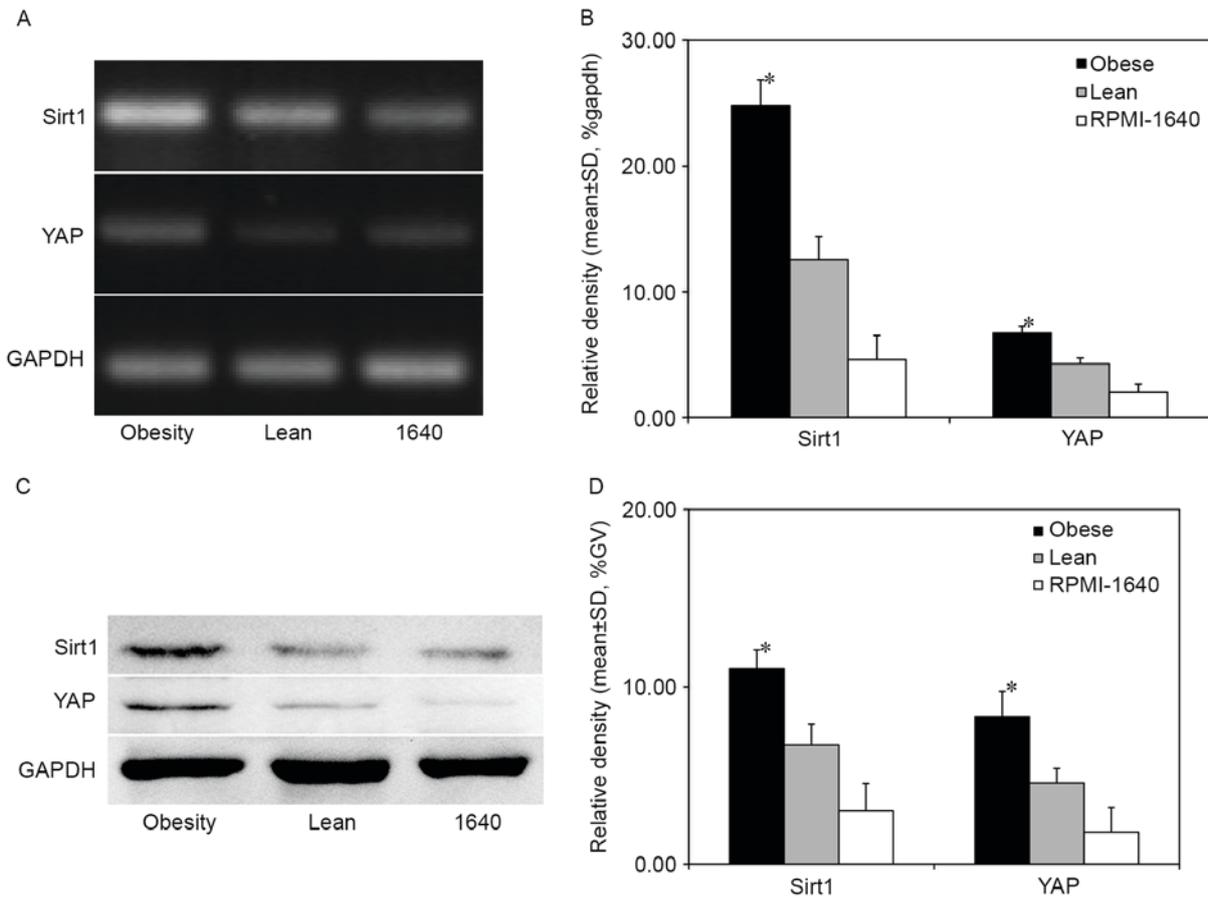


Figure 4. Obesity accelerates the growth of murine gastric cancer via the Sirt1/YAP signaling pathway at the mRNA and protein levels. (A and B) Reverse transcription-polymerase chain reaction results and (C and D) western blot analysis of Sirt1 and YAP expression in MFC cells following incubation with 5% sera from obese or lean mice. \* $P < 0.05$  vs. lean mice and the control RPMI-1640 group. Sirt1, silent mating-type information regulation 2 homolog 1; YAP, Yes-associated protein.

regulate MFC cell growth through the Sirt1/YAP pro-survival signaling pathway remains unknown and requires further investigation.

White adipose tissue is an energy store that secretes a large number of adipokines (5). Adipokines are primarily secreted by adipocytes and are small, biologically active factors that may serve significant roles in stimulating tumor growth and progression (32). Adipokines, including visfatin are implicated in cell growth, proliferation and angiogenesis (32). Visfatin acts as a NAD biosynthetic enzyme similar to Nampt and catalyzes the transfer of a phosphoribosyl group from 5-phosphoribosyl-1-pyrophosphate to nicotinamide, forming nicotinamide mononucleotide (NMN) and pyrophosphate (8). NMN is then converted to NAD by nicotinamide mononucleotide adenylyl-transferase (Nmnat) (33,34) through the NAD<sup>+</sup> salvage pathway. Increased levels of visfatin in obesity may promote gastric cancer growth and development by functioning as a NAD biosynthetic enzyme to increase the NAD<sup>+</sup> content, which directly affects the ability of Sirt1, then activates YAP to affect tumor growth and development. In the present study, the serum visfatin level was significantly increased in obese animals ( $44.3 \pm 3.6$  ng/ml) compared with lean animals ( $39.6 \pm 3.4$  ng/ml) and was associated with the tumor weight ( $r = 0.79$ ;  $P < 0.05$ ;  $n = 31$ ). This is consistent with previous suggestions that adipokines and other growth factors secreted in the context of obesity may enhance

cancer cell survival and solid tumor growth (30). Furthermore, the serum visfatin level was positively correlated with Sirt1 protein in the tumors in the present study, which suggests that visfatin may serve a role in the link between obesity and the Sirt1/YAP pro-survival pathway. YAP acts as the target and terminal effector of the Hippo signaling pathway, which regulates development and cell-contact inhibition (19), and may link obesity with the Hippo signaling pathway.

A previous study demonstrated that YAP is regulated by Sirt1-mediated deacetylation in cancer cells: Sirt1 deacetylates nuclear YAP protein, which upregulates or induces expression of pro-proliferation and anti-apoptotic genes via interactions with transcription factors, particularly TEAD (23). Cytoplasmic YAP is phosphorylated, which inactivates it and primes it for ubiquitin-mediated proteasomal degradation (26,27). Results from the present study demonstrated that obesity enhances the expression of Sirt1 and YAP protein. Future studies are required to verify the direct regulation of YAP by Sirt1.

In conclusion, the present study demonstrated that obesity potentiates transplanted tumor growth in mice, potentially through regulation of the Sirt1/YAP signaling pathway. The Sirt1/YAP signaling pathway may promote gastric cancer cell migration, proliferation and survival, and increase cell cycling through an endocrine mechanism. However, this requires future study.

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