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Original Article

Effect of *Mst1* overexpression on the growth of human hepatocellular carcinoma HepG2 cells and the sensitivity to cisplatin *in vitro*

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Mammalian STE20-like kinase 1 (Mst1) is the mammalian homologue of *Drosophila* Hippo, a major inhibitor of cell proliferation in *Drosophila*. It ubiquitously encodes serine threonine kinase that belongs to the family of protein kinases related to yeast STE20, and is involved in cell proliferation, apoptosis, oncogenesis, and organ growth. Recent studies have shown that Mst1 has tumor-suppressor function, and the deletion or mutation of Mst1 is reported to be associated with tumorigenesis. To investigate the effect of overexpression of *Mst1* on the growth of human liver cancer cell line HepG2 cells and the sensitivity to cisplatin *in vitro*, here we constructed recombinant eukaryotic expression vector pEGFP-N1-Mst1 containing *Mst1* gene, and transiently transfected into HepG2 cells. The effects of Mst1 overexpression on the cell proliferation and apoptosis, the phosphorylation status of Yes-associated protein, and the mRNA transcript levels of connective tissue growth factor (*CTGF*), amphiregulin (*AREG*), and *birc5* (*Survivin*) were determined. Results showed that overexpression of Mst1 inhibited cell proliferation, induced apoptosis of HepG2 cells, promoted YAP (Ser127) phosphorylation, and downregulated the mRNA expression of *CTGF*, *AREG*, and *Survivin*. We also investigated the relationship between the expression and cleavage of Mst1 and cisplatin-induced cell death. We found that Mst1 overexpression could induce cisplatin chemosensitivity, and cisplatin could promote the cleavage of Mst1 without affecting the expression of Mst1. Overall, our results indicated that Mst1 might be a promising anticancer target.

Keywords hepatocellular carcinoma; mammalian STE20-like kinase 1; apoptosis; Yes-associated protein; cisplatin

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Introduction

Live cancer (LC) is a digestive system cancer, the fifth most common malignant neoplasm worldwide and third most frequent cause of cancer-related death due to its highest mortality rate and recurrence rate [1]. Despite multimodal therapy, including surgery, chemotherapy, and radiotherapy, the curative effect on LC patients is not as good as anticipated and the prognosis remains poor [2]. Therefore, novel therapeutic schemes to complement or replace the existing modalities for treating LC are required. Gene therapy, which is characterized by an experimental therapeutic strategy with high efficiency, high specify, and high targeting, has been shown to have bright prospect [3].

Mammalian STE20-like kinase 1 [Mst1, also named serine threonine kinase 4 (STK4) and kinase responsive to stress 2 (KRS2)] and Mst2 are 59 kDa class II germinal centre (GC) kinases that share 76% identity in amino acid sequence [4] and are the closest mammalian homologs of the *Drosophila* Hippo protein kinase. Mice lacking either Mst1 or Mst2 were viable and fertile, but those lacking both of them were not. Mice developed massive hepatomegaly and hepatocellular carcinoma (HCC) when both *Mst1* and *Mst2* are liver-specific double knockout. Therefore, a single functional copy of *Mst1* or *Mst2* is necessary and sufficient for normal mouse development and they can substitute each other in the most essential functions in a redundant manner and serve as critical HCC tumor suppressors [5–7]. Mst1 is a serine threonine kinase, which regulates cell growth, proliferation, apoptosis, and organ size [8]. The activation of Mst1 kinase can be regulated by phosphorylation, autophosphorylation, dephosphorization, caspase cleavage, dimerization, and cofactor preference. Activated Mst1 has pro-apoptotic function via activating C-Jun N-terminal kinase and p38 mitogen-activated protein kinase (p38MAPK), phosphorylating histone 2B (Ser14), histone H2AX (Ser139), forkhead box O 3 (FOXO3) (Ser207) and FOXO (Ser212), promoting the acetylation of p53 (Lys382), and interacting with RAS association domain family 1A [8–10].

Of note, the activity of Mst1/2 is able to affect nuclear and cytoplasmic localization of Yes-associated protein (YAP) and Tafazzin (TAZ) [11,12], the mammalian Yorkie orthologs, which are the primary effectors of the mammalian Hippo

signaling pathway [13–17]. Hippo signaling pathway was first discovered in *Drosophila*. Yorkie's actions are pro-proliferative and antiapoptotic, and elimination of Yorkie is epistatic to loss of function of all of the upstream elements [18]. Hippo is the central component of this antiproliferative pathway that as a negative regulator of the oncogenic transcriptional co-activator, Yorkie, which can bind to the adaptor protein Salvador/Shar-pei, phosphorylates the Lats/Warts kinase and the non-catalytic polypeptide Mats (Mob as tumor suppressor), then phosphorylates and inhibits Yorkie, by promoting its binding to 14-3-3 and nuclear exit [13]. When Hippo signaling is attenuated, Yorkie phosphorylation is reduced or absent, leading to its nuclear localization, binding to the sequence-specific DNA-binding protein scalloped and regulation of target genes. So the loss of Hippo function results in a Yorkie-dependent accelerated proliferation, resistance to apoptosis, and massive organ overgrowth [19,20]. The *Drosophila* Hippo signaling pathway is highly conserved over evolutionary time, and the mammalian pathway has been implicated in regulating cell contact inhibition, organ size, and tumorigenesis [21,22]. YAP as a candidate oncogene is amplified and constitutively activated in many human cancers [13,23–28]. Overexpression of an activated and nuclear localized form of YAP increased mouse liver size, which ultimately resulted in HCC [13,15]. In mouse liver, combined Mst1/2 deficiency in the liver resulted in loss of inhibitory Ser127 phosphorylation of the YAP1, massive overgrowth, and HCC; however, re-expression of Mst1 in HCC-derived cell lines promoted YAP1 Ser127 phosphorylation and inactivation and abrogated their tumorigenicity, which indicated that Mst1 had tumor-suppressor function, and the deletion or mutation of *Mst1* was closely related to the tumor [6].

To investigate the biological effects of the overexpression of *Mst1* gene in human HepG2 cells *in vitro*, we constructed a recombinant eukaryotic expression plasmid containing *Mst1* gene fused with enhanced green fluorescence protein (*EGFP*) gene. We found that delivery of the *Mst1* gene promoted HepG2 cells apoptosis. Moreover, Mst1 overexpression could potentiate the antitumor effect of cisplatin against HepG2 cells. It will be helpful for the development of novel gene therapy approaches for cancer treatment.

Materials and Methods

Cell lines and cell culture

HepG2 cells cultured in Dulbecco's modified Eagle's medium medium (Solarbio, Beijing, China) plus 10% fetal calf serum (TransGen Biotech, Beijing, China), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all reagents were from Gibco BRL Life Technologies, Gaithersburg, USA). Cell lines were grown in a humidified incubator at 37°C in 5% CO_2 atmosphere, and kept free from mycoplasma contamination using plasmocin (Invitrogen, Carlsbad, USA).

Plasmid construction

The vectors [pEGFP-N1-Mst1 and pEGFP-N1 (Clontech, Mountain View, USA)] used in this study contain human cytomegalovirus (CMV) immediate early promoter. Polymerase chain reaction (PCR) used *Mst1*-length forward primer: 5′-TCAAGCTTCGAATT*ctgcac*GCCACCATGGAGACGGT ACAGCTGAG-3′; and *Mst1*-length reverse primer: 5′-CT CACCATGGTGGCG*accggt*CCGAAGTTTTGTTGCCGTC TCT-3′. The purified PCR product and transfer vector were digested, respectively, with the two enzymes, *Pst*I and *Age*I (New England Biolabs, Massachusetts, USA), at 37°C for 30 min. The enzyme-digested product was purified again with the EasyPure quick gel DNA extraction kit (TransGen Biotech) and ligated using In-Fusion™ PCR cloning kit (Clontech) at 25°C for 15 min, then at 42°C for 15 min under conditions that permitted the existence of the corresponding adhesive ends. The ligated product was used to transfect *Escherichia coli* DH5α (TransGen Biotech). Then the growing positive colonies were picked out and gently mixed with Luria-Bertani broth containing kanamycin/neomycin resistance (Kan^r). The positive clones were screened by colony PCR using universal primers CMV-forward (5′-TCTAAA-AGCTGCGGAATTGT-3′) and *Mst1*-segment reverse (5′-ATAGCTCTGGTTTTCGGAAT-3′), and the corresponding plasmids were extracted using the EndoFree plasmid kit (TransGen Biotech). Finally, the insert was confirmed by PCR as designed above and sequencing analysis with universal primers CMV-forward and pEGFP-N-3 (5′-CGTCGCCGTCCAGCTCGACCAG-3′).

Plasmid transfection

The plasmids were transfected into HepG2 cells using PolyJet™ *in vitro* DNA transfection reagent (Cat. #SL100688; SignaGen, Rockville, USA) according to the manufacturer's protocol. We got five groups in the experiments: control group (without any treatment); negative control group (transiently transfected with empty vector pEGFP-N1, named HepG2-vect); experimental group (transiently transfected with recombinant vector pEGFP-N1-Mst1, named HepG2-Mst1); positive control group [treated with 20 μg/ml cisplatin (DDP) for 24 h, named HepG2-DDP]; and combination group (transiently transfected with empty vector pEGFP-N1 or recombinant vector pEGFP-N1-Mst1 and treated with 20 μg/ml DDP for 24 h, named HepG2-vect + DDP or HepG2-Mst1 + DDP), respectively.

Cell proliferation analysis

Cell growth was determined by 3-(4,5-imethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma,

St Louis, USA). Briefly, 1×10^4 cells were seeded into a 96-well plate in quadruplicate for each condition one day before transfection. MTT reagent (20 μl) was added to each well at 5 mg/ml and incubated for another 4 h at 37°C. The formazan crystals formed by viable cells were then dissolved in 150 μl dimethyl sulfoxide (Solarbio) and measured at 490 nm for the absorbance (*A*) values. The cell inhibition rate was calculated as follows: inhibition rate = [(light absorption of the control group − light absorption of the experimental group)/light absorption of the control group] × 100%.

Hoechst 33342 staining

HepG2 cells were seeded in the 12-well plates at 1×10^5 cells/well one day before transfection. A total of 500 μl of Hoechst 33342 (Beyotime, Haimen, China) was added to each well and incubated for 30 min at 37°C in dark. Hoechst-stained nuclei were observed by using a fluorescence microscope (Olympus, Tokyo, Japan). A total of 500 cells from five random high-power fields were counted. The percent of apoptosis was expressed as ratio of apoptotic cells to total cells.

Real-time quantitative PCR

Total RNA from each cell was isolated using Trizol reagent (TransGen Biotech) according to the manufacturer's protocol. The RNA samples were treated with DNase I (Sigma), quantified, and reverse transcribed into cDNA using the TransScript first-strand cDNA synthesis SuperMix (TransGen Biotech). Real-time quantitative PCR was performed using SYBR® Premix Ex Taq™ II (Tli RNase H Plus) (TaKaRa, Dalian, China) in the ABI PRISM®7500 real-time PCR system (Applied Biosystems, Foster City, USA). The primer sequences used are shown in Table 1, and all primers were taken from the PrimerBank website (http://pga.mgh.harvard.edu/primerbank/). PCR specificity was examined by analyzing the melting curves. The data were analyzed using the comparative threshold cycle method.

Western blot analysis

HepG2 cells were washed with phosphate buffered saline (PBS) and lysed with radio immunoprecipitation assay (RIPA) cell lysis reagent containing proteinase and phosphatase inhibitors (Solarbio) at 4°C for 30 min. Cell extracts were centrifuged at 12,000 *g* for 20 min at 4°C, and the supernatants containing total proteins were mixed with an equal volume of 5 × sodium dodecyl sulfate (SDS) loading buffer. Samples were heated at 95°C for 5 min, separated by 12% gel, and then electrotransferred onto 0.22 μm PVDF membrane. The membranes were blocked in Tris-buffered saline and Tween 20 (TBST) containing 5% skimmed milk and incubated overnight with the primary antibody [obtained from Cell Signaling Technology (Beverly, USA)] specific for Mst1 (Cat. #3682, 1 : 300), YAP (Cat. #4912, 1 : 300), phospho-YAP (Ser127) (Cat. #4911, 1 : 300), or caspase-3 (Cat. #9662, 1 : 300). And then the secondary horseradish peroxidase-conjugated AffiniPure goat anti-rabbit lgG(H + L) or the secondary horseradish peroxidase-conjugated AffiniPure goat anti-mouse lgG(H + L) (1 : 10,000, ZSGB-BIO, Beijing, China) was added. An anti-β-actin monoclonal antibody (1 : 1000, ZSGB-BIO) was used as an internal control. The intensity of the western blot bands was quantified using ImageJ 1.4.3.67 Launcher Symmetry software (NIH, Bethesda, USA). The protein relative level was calculated as follows: protein relative level = (gray scale ratio between the target gene product bands and the β-actin protein bands in the experimental group)/(gray scale ratio between the target gene product bands and the β-actin protein bands in the control group).

Apoptosis assay

To examine cell apoptosis, monoplast suspension was prepared in each experimental group. All samples were stained with an Annexin V-FITC/PI apoptosis assay kit (MultiSciences, Hangzhou, China) according to the instruction manual, and analyzed with a FACScalibur flow cytometer (Becton–Dickinson, Franklin Lakes, USA).

Statistical analysis

Results are expressed as the mean ± SD. Data were compared by one-way analysis of variance (with *post hoc* Turkey's honestly significant difference test) or unpaired Student's *t*-tests using SPSS 11.5. $P < 0.05$ was considered significant.

Results

Effect of overexpression of *Mst1* on human HepG2 hepatoma cells growth and apoptosis

To examine the effect of *Mst1* overexpression on HepG2 cells, the pEGFP-N1-Mst1 recombinant plasmid [**Fig. 1(A)**] was constructed by ligating the enzyme-digested products of pEGFP-N1 and *Mst1* PCR product verified by 1% agarose gel electrophoresis analysis [**Fig. 1(B)**]. The recombinant plasmid was validated by colony PCR [**Fig. 1(C)**] and DNA sequencing [**Fig. 1(D)**], and then transiently transfected into HepG2 cells. The efficiency of transfection was ~70% displayed by EGFP after transfected for 48 h using a fluorescence microscope (**Fig. 2**), and the overexpression of fusion protein Mst1-EGFP was confirmed by western blot analysis [**Fig. 3(A)**]. MTT assays revealed that cell growth was significantly inhibited in pEGFP-N1-Mst1-vector transfected cells and 20 μg/ml DDP-treated cells compared with pEGFP-N1 vector-transfected cells, and the inhibition rate was in a time-dependent manner [**Fig. 4(A), a**].

Table 1 Primers used for RT-qPCR of gene expression

Gene	PubMed No.	Sequence (5′ → 3′)	Product size (bp)
Mst1	NM_006282	(F) CCTCCCACATTCCGAAAACCA (R) GCACTCCTGACAAATGGGTG	131
CTGF	NM_001901	(F) ACCGACTGGAAGACACGTTTG (R) CCAGGTCAGCTTCGCAAGG	195
AREG	NM_001657	(F) CTGGGAAGCGTGAACCATTTT (R) TCTGAGTAGTCATAGTCGGCTC	154
Survivin	NM_001012270	(F) AGGACCACCGCATCTCTACAT (R) AAGTCTGGCTCGTTCTCAGTG	118
β-actin	NM_001101	(F) CATGTACGTTGCTATCCAGGC (R) CTCCTTAATGTCACGCACGAT	250

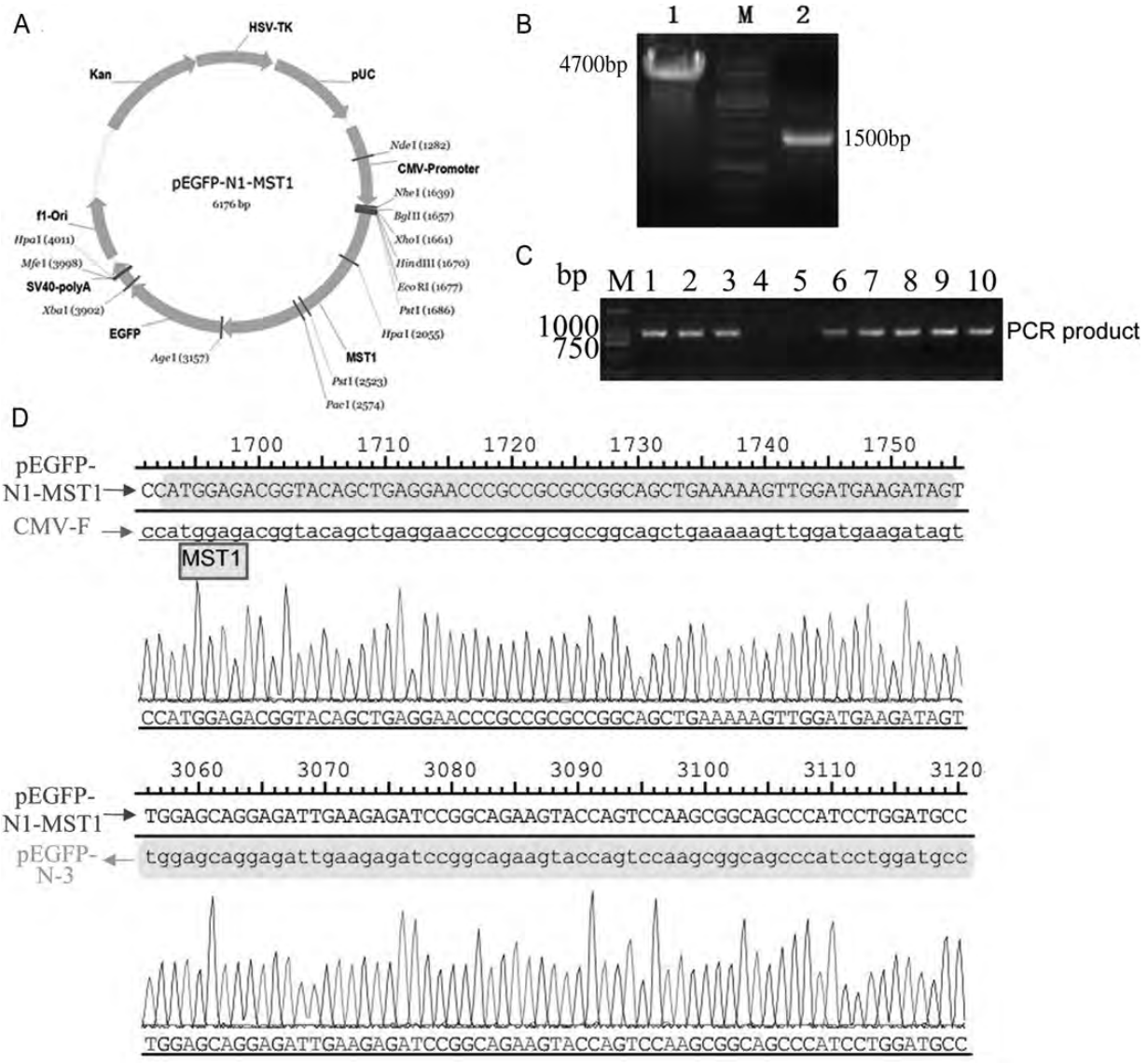

Figure 1 The construction of pEGFP-N1-Mst1 recombinant plasmid (A) The schematic diagram of pEGFP-N1-Mst1 recombinant plasmid. (B) 1% Agarose gel electrophoresis analysis of the enzyme-digested products of PCR production of Mst1 and pEGFP-N1 plasmid. M, DNA marker; line 1, pEGFP-N1 plasmid; line 2, PCR production of Mst1. (C) 1% Agarose gel electrophoresis analysis of colony PCR production. M, DNA marker; lines 1–3, 6–10, positive clones; lines 4 and 5, negative clones. (D) Partial DNA sequencing results.

Furthermore, a greater antiproliferative effect of DDP was observed when treated with 20 μg/ml DDP [**Fig. 4(A), a**]. We used annexin-V/PI staining to assess apoptosis and found that cell apoptosis was induced in pEGFP-N1-Mst1 vector-transfected cells or 20 μg/ml DDP-treated cells [**Fig. 4(B), c,d**] compared with control group [**Fig. 4(B), a**] and transfected with pEGFP-N1 [**Fig. 4(B), b**]. Moreover, after treatment with DDP [**Fig. 4(B), e**], the apoptosis rate of the pEGFP-N1-Mst1 vector-transfected HepG2 cells was higher than that of either transfected with pEGFP-N1-Mst1

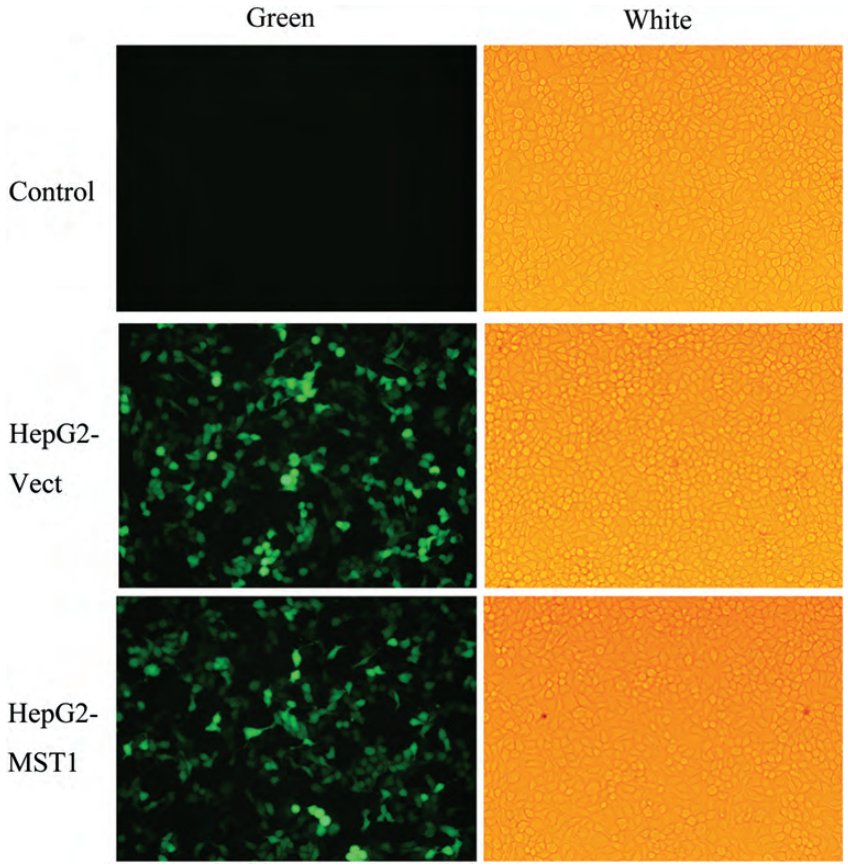

Figure 2 Transfection efficiency of HepG2 cells (magnification, ×200)

only or treated with 20 μg/ml DDP only. In addition, Hoechst 33342 staining verified that apoptotic rate was elevated to 1.5 folds compared with controls in pEGFP-N1-Mst1 vector-transfected cells and 2.0 folds over that in pEGFP-N1-Mst1 vector-transfected cells treated with DDP (**Fig. 5**). These data indicated that overexpression of *Mst1* could inhibit cell proliferation and promote apoptosis of human HepG2 hepatoma cells.

Overexpression of *Mst1* promoted YAP phosphorylation and downregulated the expression of *CTGF*, *AREG*, and *Survivin*

Levels of total YAP1 polypeptide were increased following Mst1/2 inactivation, whereas *YAP1* mRNA level was unchanged [6]. To gain insight into the effect of overexpression of *Mst1* on the Hippo signaling in HepG2 cells, we examined the phosphorylation status of YAP, the activation of Mst1 and caspase-3 [**Fig. 3(A)**], and the expression of connective tissue growth factor (*CTGF*), amphiregulin (*AREG*), and *birc5* (*Survivin*) (**Fig. 6**). The catalytically active peptides of Mst1 [**Fig. 3(B)**] and caspase-3 proteins [**Fig. 3(D)**] and YAP phosphorylation [**Fig. 3(C)**] were significantly increased in the pEGFP-N1-Mst1 vector-transfected cells, DDP-treated cells, and HepG2-Mst1 + DDP cells compared with the control group. It should be noted that DDP promoted both endogenously and exogenously expressed Mst1 to be cleaved, which led to the reduction of fusion protein Mst1-EGFP and full length (FL) Mst1, and the exaltation of Mst1 carboxy terminus (CT) [**Fig. 3(B)**]. What's more, the relative transcript levels for *CTGF*, *AREG,* and *Survivin* were remarkably reduced in these cells (**Fig. 6**). *CTGF* was downregulated 0.4 fold in pEGFP-N1-Mst1 vector-transfected cells, 0.8 fold in DDP-treated cells, and 0.95-fold in HepG2-Mst1 + DDP cells. Similar results were found for *AREG* and *Survivin*. Especially, >200-fold increase in *Mst1* transcripts was found in pEGFP-N1-Mst1 vector-transfected cells and combination group cells (**Fig. 6**). These results indicated that overexpression of *Mst1* promoted YAP phosphorylation and downregulated the expression of *CTGF*, *AREG,* and *Survivin*.

The relationship between the expression and activation of Mst1 and DDP-induced cell death

Mst1 has also been involved in the death of cancer cells treated with anticancer drugs such as DDP, a broad-spectrum anticancer and platinum-based DNA-damaging agent [29]. To investigate the relationship between the expression and cleavage of Mst1 and DDP anticancer effects,

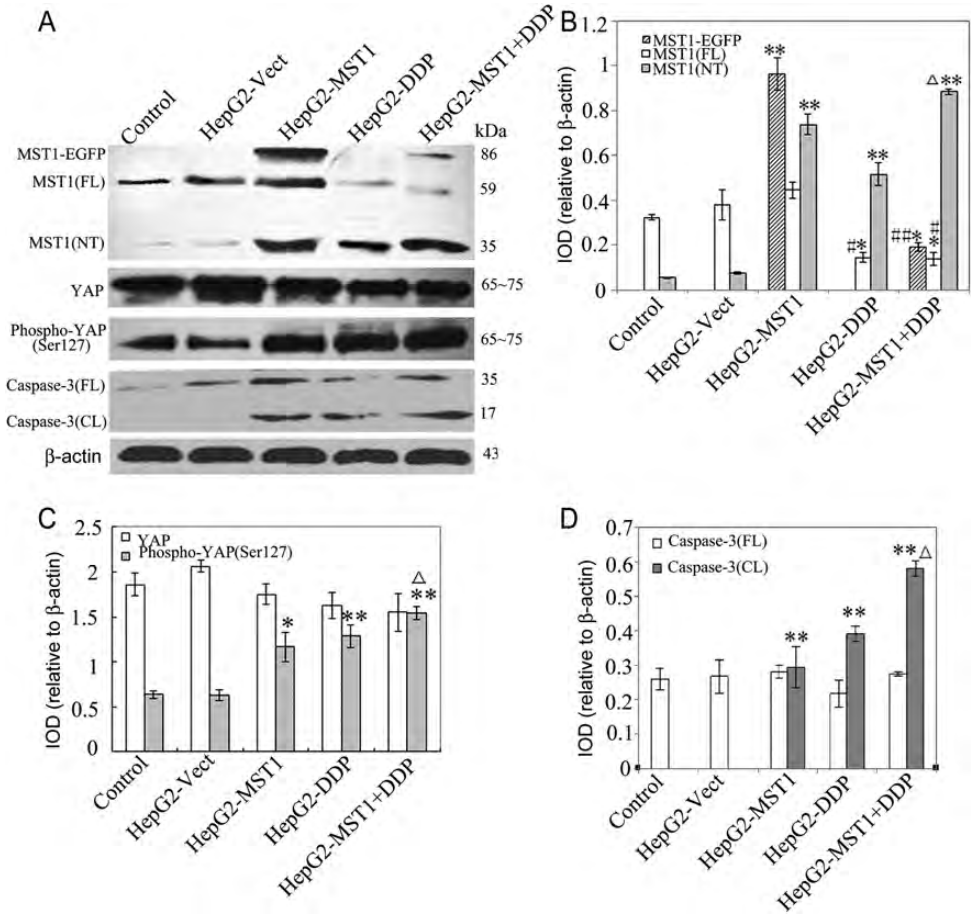

Figure 3 Western blot analysis of Mst1, YAP, phospho-YAP, and caspase-3 in each group HepG2 cells (A) Results of expression of fusion protein Mst1-EGFP, FL Mst1, Mst1 CT, YAP, phospho (Ser127)-YAP, FL caspase-3 and caspase-3 cleavage (CL) in each group HepG2 cells detected by western blot analysis. (B–D) Relative levels of fusion protein Mst1-EGFP, FL Mst1, Mst1 CT, YAP, phospho (Ser127)-YAP, FL caspase-3 and caspase-3 CL expression as assessed by gray-values. Values were normalized to that of β-actin protein. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control or HepG2-vect; $^{\triangle}P < 0.05$ vs. HepG2-Mst1 or HepG2-DDP; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. HepG2-Mst1.

firstly, we treated HepG2 cells, pEGFP-N1-Mst1 vector-transfected HepG2 cells and pEGFP-N1 vector-transfected HepG2 cells with 0, 5, 10, 15, 20, 25, and 30 μg/ml DDP *in vitro*, respectively. MTT assay [**Fig. 4(A), b**] and annexin-V/PI staining [**Fig. 7(A,C)**] showed *Mst1* overexpression increased the inhibition effect of DDP for HepG2 cells. Secondly, we treated HepG2 cells with 20 μg/ml DDP *in vitro* and analyzed the expression of Mst1, YAP, and phospho-YAP (Ser127) proteins by western blot analysis, we found that DDP elevated the levels of the catalytically active N-terminal peptide of Mst1 and the phosphorylation of YAP, and reduced the level of FL Mst1 in a time-dependent manner, but did not affect the expression of total Mst1 protein [**Fig. 7(B)**]. Therefore, it suggested that there is some relationship between the activation of Mst1 and DDP-induced cell death. *Mst1* overexpression could increase the sensitivity to DDP and it could promote the activation of Mst1 protein *in vitro*.

Discussion

In this study, we successfully constructed the eukaryotic cell expression vector containing human wild type *Mst1* gene and *EGFP* gene, and the *Mst1* gene was successfully transfected into human HepG2 cells. We found that *Mst1* overexpression could inhibit the growth of HepG2 cells, and this antiproliferation effect was associated with the induction of apoptosis. In addition, overexpression of *Mst1* gene increased the sensitivity to DDP, and DDP could promote the activation of Mst1 protein, which may imply that there is some relationship between Mst1 and DDP during DDP-induced cell death. These results showed the prospects for *Mst1* gene therapy in hepatocarcinoma treatment, also provided theoretical and experimental basis for *Mst1* gene in clinical application, but specific mechanisms need to be further discussed. Moreover, it suggested that gene delivery to HepG2 cells may allow for new

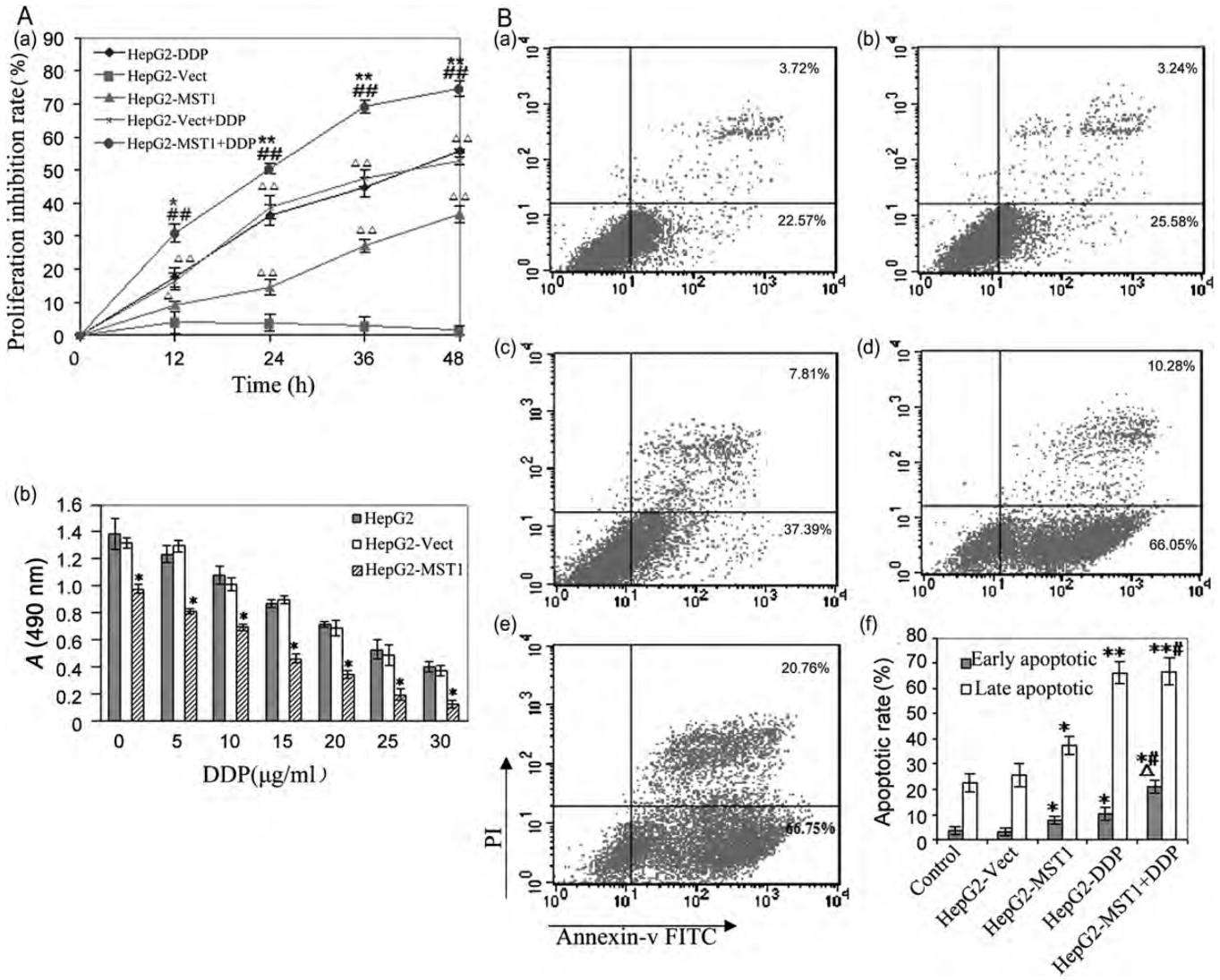

Figure 4 Overexpression of *Mst1* inhibited cell proliferation and promoted apoptosis of human HepG2 hepatoma cells (A) Cell growth was determined by MTT assay. (a) The proliferation inhibition rate of control group cells, HepG2-vect cells, HepG2-Mst1 cells, HepG2-DDP cells, HepG2-Mst1 + DDP cells, and HepG2-vect + DDP cells for 12, 24, 36, and 48 h, respectively ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. HepG2-DDP or HepG2-vect + DDP; $^{\#\#}P < 0.01$ vs. HepG2-Mst1; $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$ vs. HepG2-vect). (b) The proliferation activity of control group cells, HepG2-vect cells, and HepG2-Mst1 cells represented by the value of A_{490nm} after treated with 0, 5, 10, 15, 20, 25, and 30 μg/ml cisplatin (DDP) for 16 h, respectively ($^{*}P < 0.05$ vs. HepG2 or HepG2-vect). (B) Apoptosis rate of control group cells, HepG2-vect cells, HepG2-Mst1 cells, HepG2-DDP cells, and HepG2-Mst1 + DDP cells were detected by FACS analysis. (a) Control group. (b) Negative control group, transfected pEGFP-N1 for 48 h. (c) Experimental group, transfected pEGFP-N1-Mst1 for 48 h. (d) Positive control group, treated with 20 μg/ml DDP for 24 h. (e) Combination group, transfected pEGFP-N1-Mst1 for 24 h later, treated with 20 μg/ml DDP for 24 h. (f) The statistical analysis of the apoptosis rate. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control or HepG2-vect; $^{\#}P < 0.05$ vs. HepG2-Mst1; $^{\triangle}P < 0.05$ vs. HepG2-DDP.

therapeutic approaches to this life-threatening disease, and *Mst1* may be as a potential therapeutic target for human tumors.

The transcriptional co-activator YAP is an evolutionarily conserved regulator of organ size and progenitor of cell proliferation [24]. Morin-Kensicki *et al*. [30] found homozygosity for the $Yap^{tm1Smil}$ allele ($Yap^{-/-}$) caused developmental arrest around E8.5. What's more, Mst1 and 2 acted in a redundant manner to maintain quiescence in the adult liver and that their dual inactivation led to immediate YAP1 nuclear residence and activation, liver overgrowth, resistance to Fas-induced apoptosis, and rapid HCC development, resulting from loss of YAP1 Ser127 phosphorylation [6]. Inactivation of Mst1 and 2 in liver was accompanied by expansion of both the hepatocytes and the bipotential adult liver progenitors, and characterized by many tumors with mixed cellularity, the loss of YAP1 (Ser127) phosphorylation and a marked increase in overall and nuclear YAP1 abundance [30,31]. Moreover, Mst1 could restrain intestinal stem cell proliferation and colonic

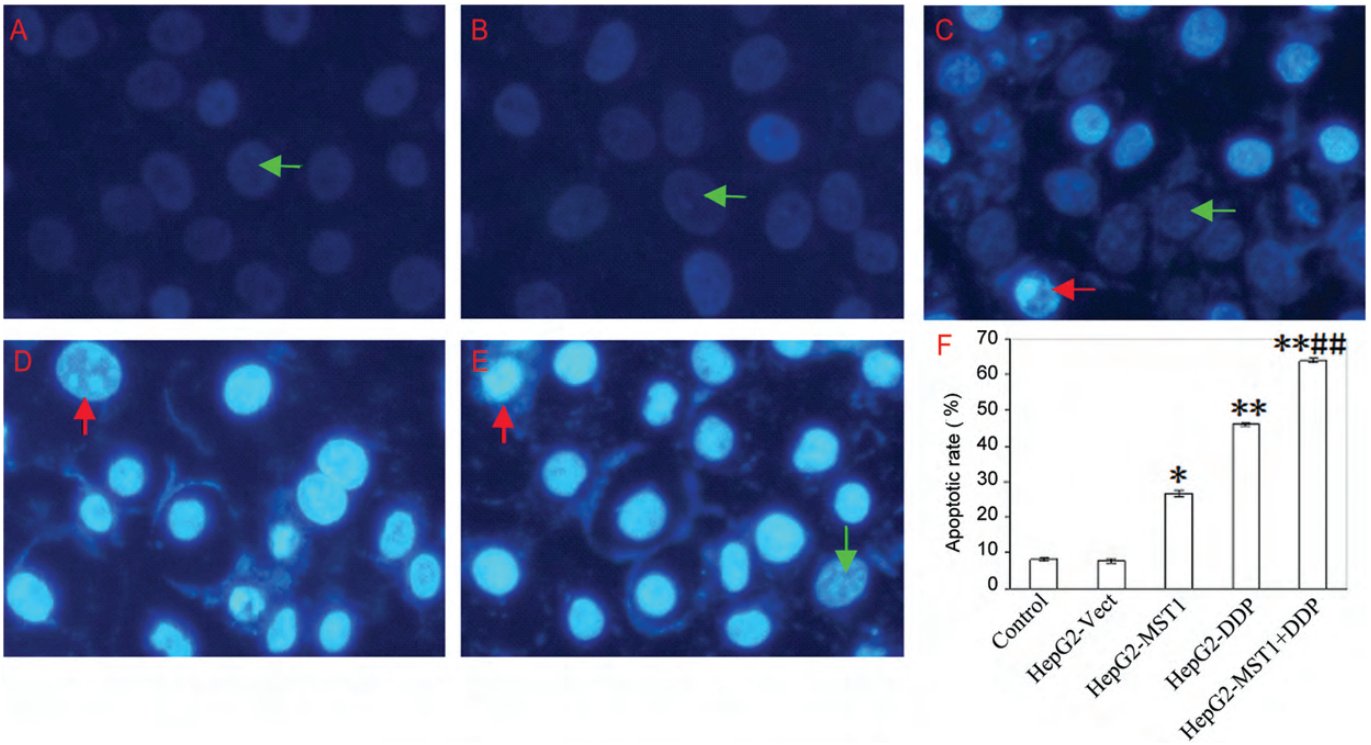

Figure 5 Hoechst 33342 staining of each group HepG2 cells (A) Control group. (B) Negative control group, transfected pEGFP-N1 for 48 h. (C) Experimental group, transfected pEGFP-N1-Mst1 for 48 h. (D) Positive control group, treated with 20 μg/ml DDP for 24 h. (E) Combination group, transfected pEGFP-N1-Mst1 for 24 h later, treated with 20 μg/ml DDP for 24 h. (F) Quantitation of Hoechst 33342 staining (*$P < 0.05$, **$P < 0.01$ vs. control or HepG2-vect, $^{\#\#}P < 0.01$ vs. HepG2-Mst1 or HepG2-DDP). The red arrows indicate apoptotic cells, and the green arrow indicates normal cells. Magnification, ×400.

tumorigenesis by inhibition of YAP overabundance [32]. Here our data showed that overexpression of Mst1 inhibited cell proliferation, increased the apoptosis of HepG2 cells *in vitro* and promoted YAP Ser127 phosphorylation and caspase-3 activation. These findings indicated that Mst1 induced apoptosis may be through the phosphorylation of YAP1 (Ser127), which resulted in restraining its activity of promoting proliferation in mammalian HepG2 cells.

In both the liver tumor nodules and pretumor mutant liver samples, *Survivin* mRNA expression was significantly upregulated [5], and knockdown of YAP enhanced the sensitivity of cancer cells to the small-molecule antagonist of survivin, S12, a lead compound which can bind to survivin and exhibit promising biological activities [31]. Both *CTGF* and *AREG* have been identified as transcriptional targets of YAP required for cell growth and phenotypes [32,33]. *Mst1/2* null liver and HCC expressed substantial levels of *CTGF* [6]. Liu *et al*. [34] reported that *Survivin* was upregulated >9 folds in savl mutant compared with wild type and >35 folds in tumor tissue, and similar observation was found for *CTGF*. Here, we found overexpression of *Mst1* significantly reduced *Survivin*, *CTGF,* and *AREG* mRNA expression. These results further supported that Mst1 regulated *Survivin*, *CTGF,* and *AREG* expression via YAP (Ser127) phosphorylation.

Mst1 has an N-terminal catalytic domain and a C-terminal regulatory region. During apoptosis, the activation of caspase-3 usually results in Mst1 cleaved to remove the C-terminal regulatory domain and yield a catalytically active N-terminal peptide cleaved by caspase at DEMD^{326}S and TMTD^{349}G sites [35,36], which correlates with an increase in Mst1 activity. Exogenously expressed Mst1 could activate caspases that resulted in its cleavage [36]. Here we found that the level of Mst1 (CT) and caspase-3 (CL) were increasing followed by the elevated expression of fusion protein Mst1-EGFP in HepG2 cells *in vitro*. We concluded that Mst1 may be as both upstream and downstream factor of caspases, and there may be a crosstalk between Mst1 and caspase-mediated apoptotic signaling.

What's more, our data showed that Mst1 could enhance the sensitivity of HepG2 cells to DDP, and DDP could promote the cleavage of both endogenously and exogenously expressed Mst1 without affecting the expression of Mst1. Morinaka *et al*. [37] found that endogenous peroxiredoxin-I (PRX-I) was required for H_2O_2-induced Mst1 activation, and DDP treatment induced H_2O_2 generation, which likewise caused PRX-I oligomer formation, Mst1 activation, and cell death. Moreover, studies found that Mst1 phosphorylated Sirtuin1 (Sirt1) and inhibited the deacetylation of Sirt1 that led to increasing the acetylation and

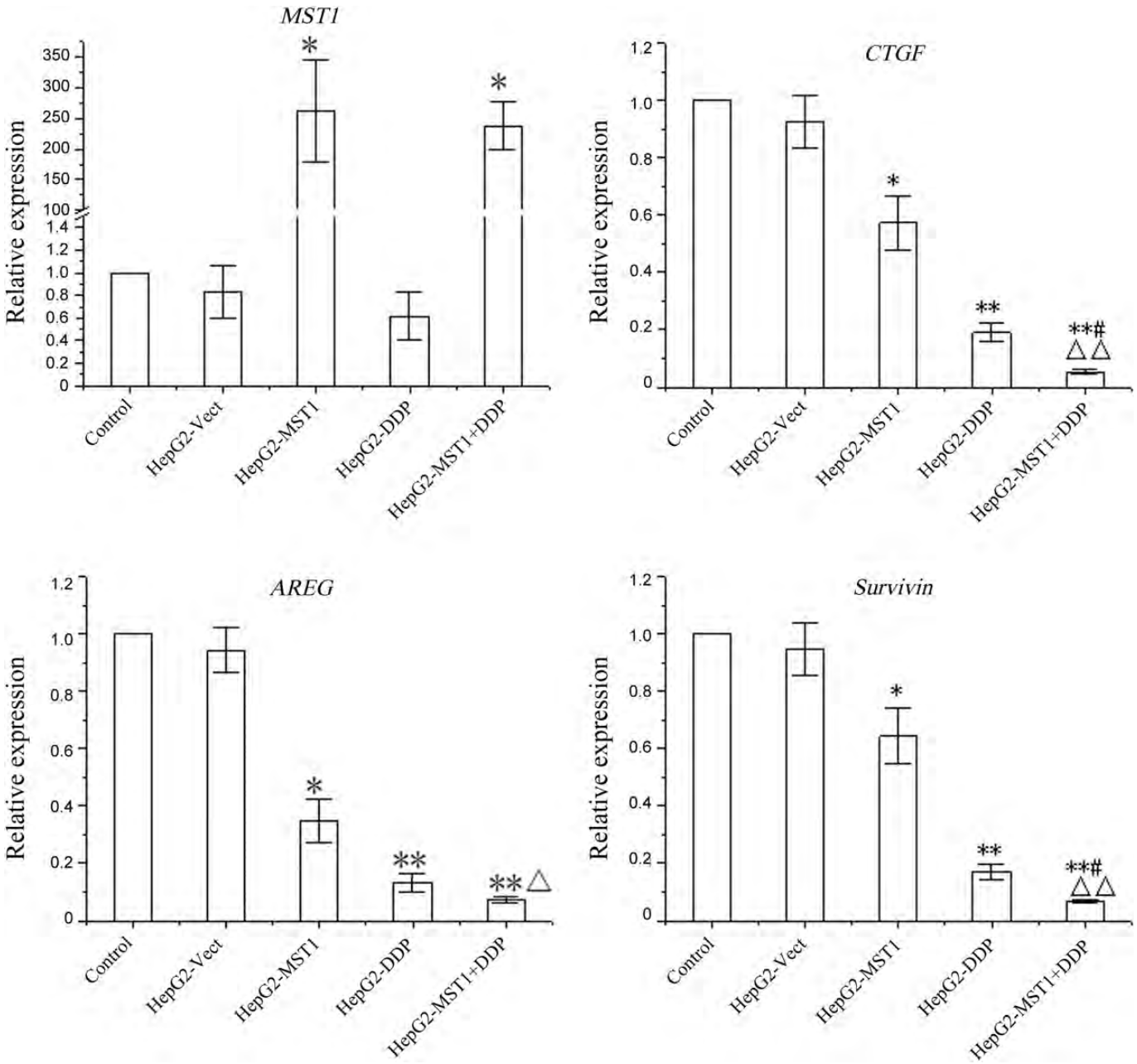

Figure 6 Quantitative RT-PCR analysis of *MST1*, *CTGF*, *AREG,* and *Survivin* mRNA expression in each group HepG2 cells $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control or HepG2-vect; $^{\#}P < 0.05$ vs. HepG2-DDP; $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$ vs. HepG2-Mst1.

transactivation of p53 and inhibiting the interaction between Sirt1 and p53 [9]. In addition, in response to DNA damage, c-Abl was activated, bound to p73 via the SH3 domain and phosphorylated p73 (Tyr99) [38], and also phosphorylated YAP1 (Tyr357). The phosphorylated YAP1 escaped the itch promoter-associated Runx [39]. YAP1 bound to p73 through its WW domain and the PPPY motif of p73, and promoted the transcriptional activity of p73 via the p300 acteyltansferase and the pro-myelocytic leukemia (PML) tumor-suppressor protein [40–42], which resulted in the selective co-activation of the p73 pro-apoptotic targets, such as p53 regulated apoptosis inducing protein 1 (*p53 AIP1*), BCL2-associated X protein (*Bax*), and p53 inducible protein 3 (*PIG3*) [43]. In addition, YAP1 could stabilize p73 protein in a post-translational manner through competing with the itch E3-ligase for binding to p73, which allowed p73 to escape itch-mediated ubiquitination [44]. In summary, under DNA damage, YAP1 is a pro-apoptotic factor forming a complex with p73 and PML to regulate pro-apoptotic targets like *p53 AIP1* and *Bax*. Therefore, it may imply that

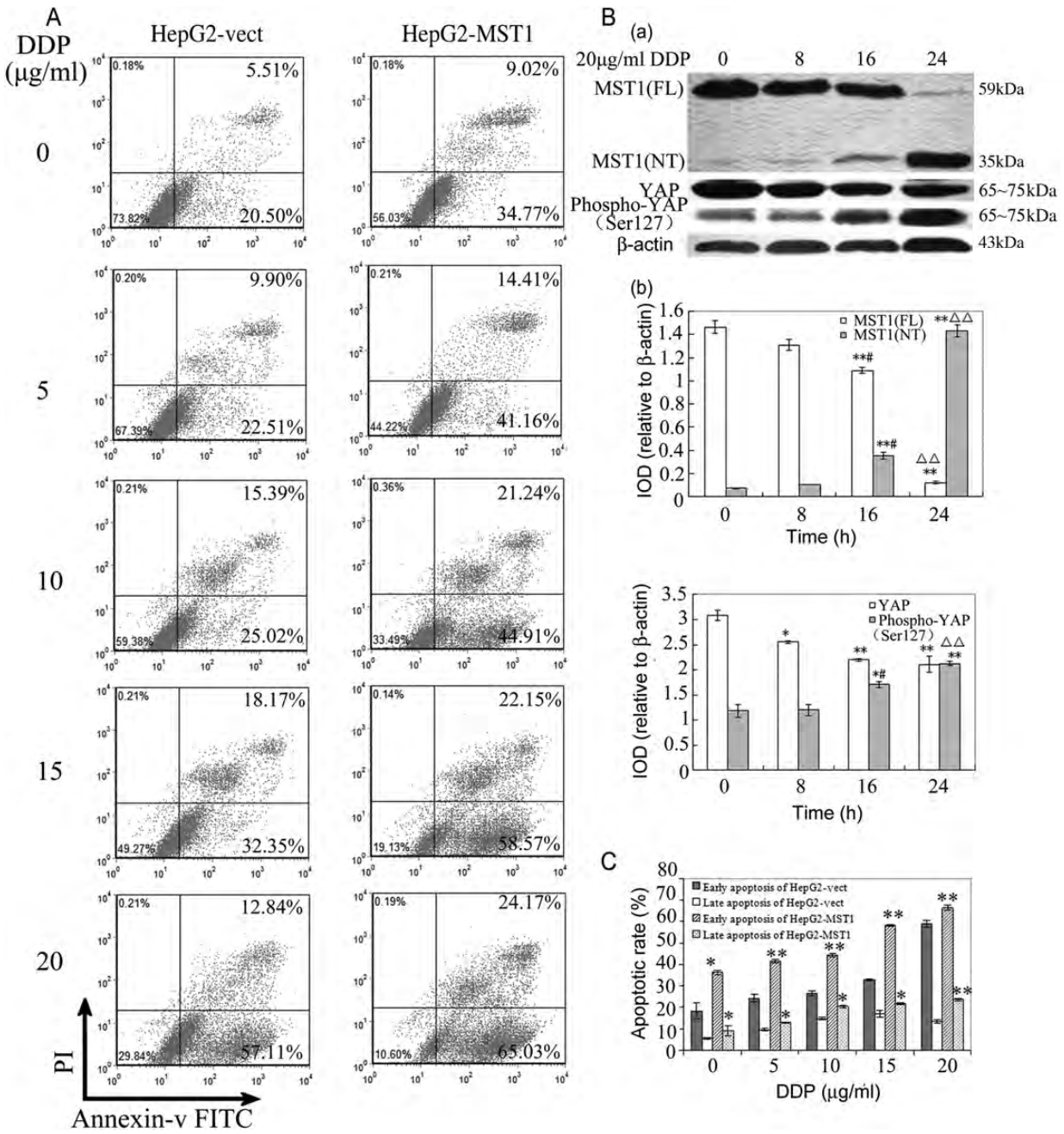

Figure 7 The effect of MST1 overexpression on the sensitivity to cisplatin *in vitro* (A) Apoptosis rates of pEGFP-N1-Mst1 vector-transfected HepG2 cells and pEGFP-N1 vector-transfected HepG2 cells treated with 0, 5, 10, 15, 20 μg/ml cisplatin for 16 h were detected by FACS analysis. (B) Western blot analysis of Mst1, YAP, and phospho-YAP (Ser127) in HepG2 cells treated by cisplatin for 0, 8, 16, 24 h. (a) Results of expression of FL MST1, MST1 CT, YAP, and phospho (Ser127)-YAP proteins in HepG2 cells treated by cisplatin for 0, 8, 16, 24 h detected by western blot analysis. (b) Relative levels of FL Mst1, Mst1 CT, YAP, and phospho (Ser127)-YAP proteins expression as assessed by gray-values. Values were normalized to that of β-actin protein. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. 0, 8 h; $^{\#}P < 0.05$ vs. 8 h, $^{\triangle\triangle}P < 0.01$ vs. 16 h. (C) The statistical analysis of the apoptosis ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. early apoptosis of HepG2-vect or late apoptosis of HepG2-vect).

there is probably some relationship among DDP, Mst1, YAP1, p53, and p73 during DDP-induced cell death (**Fig. 8**). *Mst1* gene may be a useful candidate for the chemotherapy. However, additional studies exploring the *in vivo* biological activities of Mst1 are needed in the future, which will support Mst1 as a potential target for cancer therapy.

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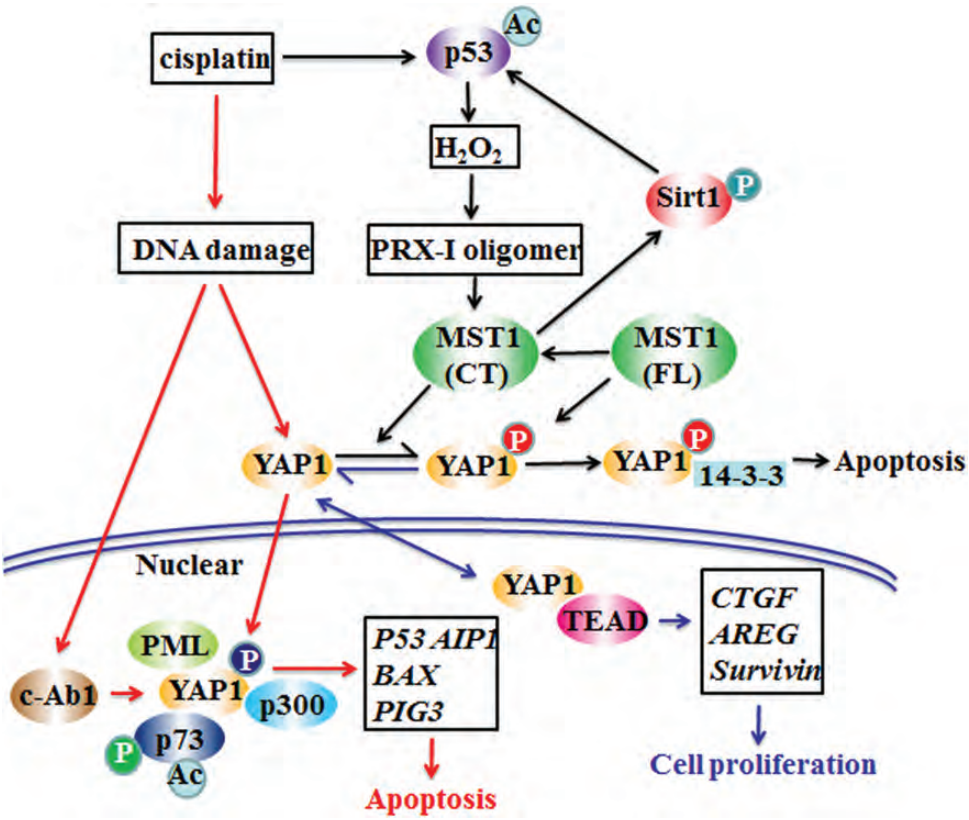

Figure 8 Diagram for the relationship between cisplatin, Mst1, YAP1, p53, and p73 during cisplatin-induced cell death On the one hand, during the cisplatin-induced cell death, cisplatin causes the increase of p53, H_2O_2, peroxiredoxin-I (PRX-I) oligomer, and Mst1 kinase activity in turn. Activated Mst1 can phosphorylate Sirtuin1 (Sirt1) which leads to p53 actylation. Both FL Mst1 and Mst1 CT can promote YAP (Ser127) phosphorylation that led to its binding to 14-3-3 and downregulated the expression of *CTGF*, *AREG,* and *Survivin*. On the other hand, under DNA damage, c-Abl is activated. Activated c-Abl binds to p73 and phosphorylates p73 (Tyr99), and it also phosphorylates YAP1 (Tyr357). YAP1 binds to p73 via the p300 acteyltansferase and the PML tumor-suppressor protein to induce transcription of p73 pro-apoptotic target genes like p53-regulated apoptosis inducing protein 1(*p53 AIP1*), BCL2-associated X protein (*Bax*) and p53 inducible protein 3 (*PIG3*).

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