The RING domain in the anti-apoptotic protein XIAP stabilizes c-Myc protein and preserves anchorage-independent growth of bladder cancer cells

Guosong Jiang‡§1, ‡ Chao Huang‡§1, Xin Liao§1, Jingxia Li§, Xue-Ru Wu§, Fuqing Zeng‡2, and Chuanshu Huang‡3

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The incidence and mortality rate of bladder cancer (BC)4 rank in the first place in urologic malignancies and have continued to rise in recent years. There are more than 429,000 new cases of BC diagnosed every year worldwide (1), and about 81,190 patients were diagnosed in the United States in 2018 (2). Treatment based on the grade and stage of BC ranges from transurethral resection to radical cystectomy to systemic chemotherapy. Currently, the overall therapeutic effects on muscle-invasive BC are limited, and the 5-year survival rate has remained at a low level (3). Thus, further exploration of genetic regulatory networks involved in BC progression and development of precise strategies are of great significance.

X-linked inhibitor of apoptosis protein (XIAP), a member of the inhibitor of apoptosis (IAP) family, contains three baculoviral IAP repeat (BIR) domains and a ubiquitin-associated RING domain (4). XIAP not only functions as a suppressor of apoptosis, but also plays key roles in development, anchorage-independent growth, migration, and invasion of cancer cells, as well as mediating resistance of cancer cells to chemotherapeutic drugs and radiotherapy (5). Recently, we found that XIAP promoted urothelial transformation through its C-terminal RING domain–initiated miR-4295 expression and downstream reduction of p63α protein translation (6). On the other hand, we previously have reported that the RING domain of XIAP enhances BC cell anchorage-independent growth through up-regulating cyclin D1 expression (7), and we also have revealed that the XIAP N-terminal BIR domain increases EGFR translation and promotes growth of BC cells (8). Moreover, we recently discovered that XIAP promoted BC invasion in vitro and lung metastasis in vivo through enhancing nucleolin–mediated Rho-GDIβ mRNA stability (9). Judging from the multiple roles of XIAP in tumor formation and progression, it may serve as a promising target for BC therapy.

The oncoprotein c-Myc plays key roles in formation and progression of various cancers, and it is reported that c-Myc could regulate expression of about 15% of all genes through binding to their promoters and enhancers (10). The expression of activation targets of c-Myc, an embryonic stem signature, was observed more frequently in poorly differentiated BC than in well-differentiated ones (11). When human BC cell lines with low versus high metastatic potential are compared, c-myc is found to be up-regulated in the highly metastatic tumor cell lines (12). In the present study, we explore the mechanism underlying increased c-Myc expression in muscle-invasive

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1 These authors contributed equally to this work.
2 To whom correspondence may be addressed: Dept. of Urology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China, and the 3Department of Urology, New York University School of Medicine, New York, New York 10016
3 To whom correspondence may be addressed: Nelson Institute of Environmental Medicine, New York University School of Medicine, Tuxedo, New York 10987, the 4Department of Urology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China, and the 5Department of Urology, New York University School of Medicine, New York, New York 10016

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bladder cancer cells and focus on the correlation between up-regulation of c-Myc and overexpression of XIAP. We found that the XIAP RING domain could stabilize c-Myc protein through inhibition of its phosphorylation at Thr-58, which is mediated by increased phosphorylation of glycogen synthase kinase-3β (GSK-3β) at Ser-9 due to ERK1/2 activation. Moreover, c-Myc is an effector for promotion of anchorage-independent growth and invasion by XIAP RING domain in BC cells.

Results

XIAP RING domain, but not BIR domain, is crucial for XIAP-mediated up-regulation of c-Myc protein expression

To investigate the correlation between up-regulation of c-Myc and overexpression of XIAP, shRNAs specifically targeting human XIAP RING domain (shXIAP77) and specifically targeting human XIAP BIR domain (shXIAP78) mRNA coding areas were used to knockdown endogenous XIAP in two different invasive bladder cancer cell lines, T24T and UMUC3, respectively (Fig. 1, A and B). XIAP RING domain–negative (ΔRING) and its scramble vector control (pEEB) plasmids were then co-transfected with shXIAP77, and XIAP BIR domain–negative (ΔBIR) and its scramble vector control (pEEB) plasmids were co-transfected with shXIAP78 (thus avoiding knockdown of the exogenous RING domain of XIAP by its shRNA) to rescue the expression of the BIR and RING domains, respectively, in these knockdown transfectants. The stable transfectants were established and analyzed for c-Myc expression. As shown in Fig. 1 (C and D), knockdown of XIAP by shXIAP77 or shXIAP78 in both cell lines resulted in a profound reduction of c-Myc protein levels in both T24T and UMUC3 cells, which were effectively reversed by ectopic expression of RING (ΔRING domain, but not BIR (ΔBIR) domain, but not BIR (ΔRING) domain. However, the mRNA levels of c-Myc were not affected in these stable transfectants (Fig. 1, E and F). We also used XIAP RING domain deletion knock in (ΔRING) and WT mice to establish both WT mouse embryonic fibroblasts (MEFs) and ΔRING MEFs. The results obtained from comparison of c-Myc protein levels in both T24T and UMUC3 cells, which were effectively reversed by ectopic expression of RING (ΔBIR) domain, but not BIR (ΔRING) domain. However, the mRNA levels of c-Myc were not affected in these stable transfectants (Fig. 1, E and F). We also used XIAP RING domain deletion knock in (ΔRING) and WT mice to establish both WT mouse embryonic fibroblasts (MEFs) and ΔRING MEFs. The results obtained from comparison of c-Myc expression between ΔRING MEFs and WT MEFs indicated that c-Myc protein expression in XIAP RING domain–deficient MEFs (ΔRING) was impaired as compared with that in WT-MEFs (Fig. 1G). Moreover, when RING domain was constitutively expressed in ΔRING MEFs, c-Myc protein level was also restored (Fig. 1H). Similar to what was observed in BC cells, neither RING domain deletion nor its ectopic expression affected c-Myc mRNA levels in MEFs (Fig. 1, I–L).

Figure 1. XIAP RING domain is required for c-Myc protein expression without alteration of its mRNA. A and B, the shRNAs (shXIAP77 and shXIAP78) were used to knock down XIAP expression in T24T and UMUC3 cells, and the stable transfectants were then subjected to Western blotting for determination of XIAP expression. C and D, the cell protein extracts obtained from the indicated stable transfectants were subjected to Western blotting for determination of expression of XIAP and c-Myc. GAPDH was used as the protein-loading control. E and F, the c-myc mRNA expression level was evaluated by RT-PCR and quantitative RT-PCR in UMUC3 cell transfectants as indicated, and gapdh mRNA was used as the internal loading control. G and H, the expression of c-Myc protein was compared in XIAP RING domain WT MEFs (WT) versus RING domain–deficient MEFs (ΔRING) (G) and in ΔRING versus ΔRING/GFP-RING MEFs (H). GAPDH was used as the protein-loading control. I–L, the c-myc mRNA expression level was evaluated by RT-PCR and quantitative RT-PCR in WT versus ΔRING MEFs (I and J) and in ΔRING versus ΔRING/GFP-RING MEFs (K and L). The gapdh mRNA was used as the internal loading control. *, significant difference as compared with nonsense cells (p < 0.05). #, significant difference as compared with vector transfectant (p < 0.05). Error bars, S.D.
XIAP RING domain stabilizes c-Myc protein through inhibiting its phosphorylation at Thr-58

To define whether XIAP regulates c-Myc protein via proteasome-mediated degradation, UMUC3 cells stably transfected with control nonsense or shXIAP78 were pretreated with or without MG132 (20 μM) for 12 h and then treated with CHX (100 μg/ml) at the indicated times. The cell extracts were subjected to Western blotting for determination of the degradation of the indicated protein. GAPDH was used as the protein-loading control.

**Figure 2. XIAP RING domain stabilizes c-Myc protein through inhibiting the phosphorylation of c-Myc at Thr-58.** A, UMUC3 (nonsense) and UMUC3 (shXIAP78) stable transfectants were pretreated with or without MG132 (20 μM) for 12 h and then treated with CHX (100 μg/ml) at the indicated times. The cell extracts were subjected to Western blotting for determination of the degradation of the indicated protein. GAPDH was used as the protein-loading control. B, UMUC3 (nonsense) and UMUC3 (shXIAP78) stable transfectants were treated with CHX (100 μg/ml) alone or co-treated with MG132 (20 μM) for 12 h, and the cell extracts were subjected to Western blotting for determination of the degradation of c-Myc protein. C, UMUC3 (nonsense) and UMUC3 (shXIAP78) stable transfectants were treated with MG132 (20 μM) for 12 h, and the cell extracts were subjected to Western blotting for determination of the level of c-Myc protein by running the SDS-PAGE on the same gel. D, UMUC3 (shXIAP78/pEBB) and UMUC3 (shXIAP78/ΔBIR) stable transfectants were pretreated with or without MG132 (20 μM) for 12 h, and then treated with CHX (100 μg/ml) at the indicated times. The cell extracts were subjected to Western blotting for determination of the degradation of the indicated protein. E–G, the indicated stable transfectants were subjected to Western blotting for determination of the level of c-Myc phosphorylation at Thr-58 and Ser-62, as well as expression of FBW7. GAPDH was used as the protein-loading control. *, significant difference as compared with nonsense cells (p < 0.05). #, significant difference as compared with vector transfectant (p < 0.05). Error bars, S.D.
Activation of ERK1/2 by the XIAP RING domain mediated phosphorylation of GSK-3β at Ser-9 and in turn inhibited c-Myc phosphorylation at Thr-58

Because GSK-3 activity has been reported to play a key role in inhibiting phosphorylation of c-Myc specifically at Thr-58 and to increase c-Myc stability (16), we next tested whether changes in the activity of GSK-3 were involved in inhibition of c-Myc phosphorylation at Thr-58 by the XIAP RING domain. As shown in Fig. 3A, knockdown of XIAP by shXIAP77 or shXIAP78 in UMUC3 cells effectively suppressed GSK-3β phosphorylation at Ser-9, with no effect on total protein level of GSK-3β, whereas both the phosphorylation status and total protein level of GSK-3β were not changed. Given that the phosphorylation level of GSK-3β at Ser-9 is negatively associated with its kinase activity (17), suppression of phosphorylation of GSK-3β at Ser-9 revealed that GSK-3β activity was increased in XIAP knockdown transfectants. Moreover, restoration of the RING domain, rather than the BIR domain, resulted in a marked increase of GSK-3β phosphorylation at Ser-9 in XIAP knockdown cells (Fig. 3A). In line with these findings, compared with WT MEFs, phosphorylation of GSK-3β at Ser-9 was decreased in XIAP RING domain–deficient MEFs (Fig. 3B) and was up-regulated upon restoration of the RING domain (Fig. 3C). To further confirm the role of GSK-3β in XIAP RING domain regulation of c-Myc phosphorylation at Thr-58, shXIAP78/ΔBIR cells were transfected with the constitutive active mutant of GSK-3β (GSK-3βS9A) into RING domain–restored UNUC3 (shXIAP78/ΔBIR) cells, and the stable transfectants were identified, as shown in Fig. 3D. The dominant active effect was proved by the promotion of a GSK-3β–regulated phosphorylation of β-catenin at Ser-33, Ser-37, or Thr-41 (Fig. 3D), as reported in previous studies (18). We found that the active form of GSK-3β could effectively enhance phosphorylation of c-Myc at Thr-58 to down-regulate its total protein expression, whereas phosphorylation at Ser-62 was also not affected (Fig. 3D).

Our previous study has demonstrated that extracellular signal–regulated protein kinase (ERK1/2) activation could be promoted by XIAP in T24T cells (9), and ERK1/2 has been reported to be the upstream kinase of GSK-3β (19). We therefore determined the potential effect of ERK1/2 on the regulation of phosphorylation of c-Myc. As shown in Fig. 4A, knockdown of XIAP by shXIAP77 or shXIAP78 in T24T cells effectively inhibited ERK1/2 phosphorylation without affecting its total protein levels. Similarly, phosphorylation of ERK1/2 was down-regulated in XIAP RING domain–deficient MEFs (Fig. 4B) and was increased upon restoration of RING domain expression (Fig. 4C). To further address the role of ERK1/2 in XIAP RING domain regulation of c-Myc phosphorylation at Thr-58, we transfected the dominant negative form of ERK2 (DN-ERK2) into RING domain–restored UNUC3 (shXIAP78/ΔBIR) cells, and the stable transfectants were identified, as shown in Fig. 4D. The dominant negative effect was observed...
with the inhibition of an ERK-regulated phosphorylation of p-P90RSK at Thr-359/Ser-363 (Fig. 4D), as reported previously (20). Ectopic expression of DN-ERK2 could effectively inhibit the phosphorylation level of GSK-3β/H9252 at Ser-9 and enhanced phosphorylation of c-Myc at Thr-58 to further down-regulate its total protein expression, whereas phosphorylation at Ser-62 was not affected (Fig. 4, D–G). These results clearly indicate that activation of ERK1/2 by the XIAP RING domain mediates phosphorylation of GSK-3β at Ser-9, which further inhibits c-Myc phosphorylation at Thr-58. On the other hand, no direct interaction of XIAP and c-Myc was observed as we used the lysates from WT and ΔXIAP HCT116 cells that were co-immunoprecipitated with anti-XIAP antibody, and immunoprecipitates were then subjected to Western blotting for determination of c-Myc protein (Fig. 4H).

RING domain is involved in preserving the XIAP-regulated anchorage-independent growth and invasion abilities of BC cells

XIAP has been reported to play key multiple roles in development, anchorage-independent growth, migration, and invasion of cancer cells, and we have recently found that the XIAP RING domain can promote urothelial transformation (22). To address the specific functions of the XIAP RING domain in BCs, UMUC3 (shXIAP78/pEBB) and UNUC3 (shXIAP78/ΔBIR) were applied to the anchorage-independent growth assay, and T24T (shXIAP78/pEBB) and T24T (shXIAP78/ΔBIR) were applied to the transwell migration and invasion assay in the present study. As shown in Fig. 5 (A–D), stable knockdown of XIAP blocked anchorage-independent growth in UMUC3 and T24T cells, which was remarkably reversed by restoration of the RING domain (ΔBIR). Accordingly, both the relative migration and invasion rates of UMUC3 cells were reduced by knockdown of XIAP, which was resumed by restoration of the RING domain (Fig. 5, E–G). Although the migration of T24T cells was even promoted by knockdown of XIAP, the relative invasion rates of T24T cells were also inhibited by knockdown of XIAP and restored by transfection of the RING domain (Fig. 5, H–J). These results indicate that the RING domain is involved in preserving the XIAP-regulated anchorage-independent growth and invasion abilities of BC cells.

c-Myc is required for promotion of anchorage-independent growth and invasion by the XIAP RING domain in BC cells

To explore the functions of up-regulation of c-Myc by the XIAP RING domain in the promotion of anchorage-independent growth and invasion in BC cells, c-Myc expression vector was constitutively overexpressed in UMUC3 (shXIAP78) and
XIAP RING domain stabilization of c-Myc

T24T (shXIAP78) cells. The stable transfectants were identified as shown in Fig. 6 (A and B) and then applied to the anchorage-independent growth assay and transwell migration and invasion assay. The anchorage-independent growth ability of XIAP knockdown cells was resumed upon c-Myc overexpression in UMUC3 (shXIAP78) and T24T (shXIAP78) cells (Fig. 6, C–F), and the cell migration and invasion abilities were restored by ectopic expression of c-Myc in UMUC3 (shXIAP78) cells (Fig. 6, G–J). These results suggest that a c-Myc defect is responsible for the reduction of anchorage-independent growth and invasion caused by XIAP knockdown. To further test this notion, we knocked down c-Myc in UMUC3 (shXIAP/ΔBIR) cells, and the stable transfectant, UMUC3 (shXIAP/ΔBIR/sh-c-Myc), and its vector transfectant, UMUC3 (shXIAP/ΔBIR/nonsense), were established and identified as shown in Fig. 7A. The results obtained from the anchorage-independent growth assay revealed that effective knockdown of c-Myc by sh-c-Myc-2 and sh-c-Myc-4 could abolish RING domain–mediated BC cell anchorage-independent growth (Fig. 7, B and C), migration, and invasion (Fig. 7, D–F). Collectively, our results strongly indicate that XIAP RING-mediated up-regulation of C-Myc is crucial for RING domain promotion of anchorage-independent growth and invasion in human BC cells.

Discussion

XIAP possesses three BIR domains in the N terminus and one RING domain in the C terminus. XIAP BIR domains mainly interact with caspases to regulate cell apoptosis, whereas the RING domain of XIAP can function as an E3 ligase to bind to caspase-3 and mitochondrial XIAP inhibitor SMAC/Diablo, as well as mediate the proteosomal degradation of itself (23, 24). Recently, the E3 ubiquitin ligase function of the XIAP RING domain has been further studied. XIAP has been found to be a ubiquitin E3 ligase for Mdm2 to promote its degradation (25). It is reported that the XIAP RING domain controls the protein stability of Cdc42 through directly conjugating poly-ubiquitin chains to the lysine 166 of Cdc42 (26). XIAP could also ubiquitinate a highly conserved Lys residue in adenylyl cyclase isoforms and thereby accelerate their endocytosis and degradation (27). In this study, we found that the XIAP RING domain stabilized c-Myc protein without direct interaction. Thus, the regulation of c-Myc protein by the XIAP RING domain might be through an indirect regulatory mechanism.

The c-Myc oncogene is a “master regulator” in development of many tumors. Amplified copies of c-Myc may play a key role in the progression of highly metastatic prostate cancer and cutaneous melanoma (28). In contrast, highly metastatic melanoma and bladder carcinoma cells did not show amplified c-myc gene, although it is overexpressed in these cancers (12). Therefore, other mechanisms may be involved in up-regulation of c-myc in BC. Previous studies have shown that the dual c-myc P1/P2 promoters are important for its transcription, which involves multiple positively and negatively acting transcription factors and signaling pathways (29, 30). A recent study also shows that the translation of c-Myc is promoted by its mRNA N6-methyladenosine nucleotide modification in human myeloid leukemia cells (31). Two phosphorylation sites of c-Myc
Figure 6. Restoration of c-Myc expression reversed the down-regulation of anchorage-independent growth and invasion abilities in XIAP-deficient cells. A and B, c-Myc was stably overexpressed in XIAP knockdown UMUC3 (A) and T24T (B) cells, and the stable transfectants were then subjected to Western blotting for identification of c-Myc expression. GAPDH was used as the protein-loading control. C–F, the indicated transfectants were subjected to an anchorage-independent growth assay. G–I, the indicated transfectants were subjected to a migration and invasion assay. *, significant difference as compared with nonsense cells (p < 0.05). #, significant difference as compared with vector transfectant (p < 0.05). Error bars, S.D.

Figure 7. Knockdown of c-Myc exhibited an inhibitory effect on XIAP-RING domain–promoted anchorage-independent growth and invasion abilities of bladder cancer cells. A, the shRNA was used to knock down c-Myc expression in UMUC3 (shXIAP78) cells, and the stable transfectants were then subjected to Western blotting for determination of the indicated c-Myc expression. GAPDH was used as the protein-loading control. B and C, the indicated transfectants were subjected to an anchorage-independent growth assay. D–F, the indicated transfectants were subjected to migration and invasion assays. *, significant difference as compared with vector transfectant (p < 0.05). #, significant difference as compared with nonsense cells (p < 0.05). Error bars, S.D.
protein, Thr-58 and Ser-62, exhibit opposing roles in the control of c-Myc protein stability (32). It has been shown that GSK3-induced c-Myc phosphorylation at Thr-58 is critical for ensuring its transient and timely protein degradation (33). In the present study, we discovered that inhibition of Thr-58 phosphorylation, which was mediated by increased phosphorylation of GSK-3β at Ser-9, was critical for stabilization of c-Myc protein by the XIAP RING domain. Our study provides novel evidence that protein degradation plays an important role in the regulation of c-Myc expression in BCs.

GSK3β is a multifunctional serine/threonine kinase that participates in a diverse array of cell functions (34). Phosphorylation of GSK3β at Ser-9 leads to its inactivation by proteasomal degradation and has been connected to many pathological conditions, including cancer (35). Several kinase-driven pathways phosphorylate GSK3β at Ser-9, including protein kinase A, protein kinase B/Akt, p90 ribosomal S6 kinase/mitogen-activated protein kinase–activating protein (p90RSK/MAPKAP), and p70 ribosomal S6 kinase (p70S6K) (19, 36). Our previous study indicated that ERK1/2 activation could be promoted by XIAP in T24T cells (9). Our current studies indicate that the XIAP RING domain was crucial for ERK1/2 activation and GSK phosphorylation at Ser-9 in human BC cells, which further mediated the c-Myc protein phosphorylation at Thr-58 and BC cell anchorage-independent growth and invasion. Although the kinase-driven pathway that is responsible for XIAP RING domain-induced ERK1/2 activation still needs to be addressed, the discovery of the XIAP RING domain in promotion of GSK-3β phosphorylation at Ser-9 provides new insight into the function of XIAP in BC tumor biology.

In summary, we found that the XIAP RING domain, but not the BIR domain, is crucial for XIAP-mediated up-regulation of c-Myc protein expression. Mechanistically, the XIAP RING domain could stabilize c-Myc protein through inhibition of its phosphorylation at Thr-58, which is mediated by increasing phosphorylation of GSK-3β at Ser-9. The results from functional studies reveal that c-Myc is a crucial effector responsible for XIAPRINGdomain-mediatedpromotionofanchorage-independent growth and invasion in BC cells. Our study uncovers a novel function of the RING domain of XIAP in regulating the c-Myc stability, further offering new theoretical support for using the XIAP RING domain and c-Myc as targets for BC cancer therapy.

Experimental procedures

Cell culture and reagents

UMUC3 cells and MEF cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% FBS, and T24T cells were cultured in RPMI1640/F12 medium (Invitrogen) with 5% FBS. All cells were maintained in a humidified incubator at 37 °C, with a 5% CO₂ atmosphere. The antibodies were purchased as follows: XIAP polyclonal antibody (BD PharMingen, San Diego, CA); GFP, β-catenin, p-β-catenin, hexokinase II, GSK3α, GSK3β, p-GSK3α, p-GSK3β, FBW7, V5, ERK1/2, p-ERK1/2, and p-P90RSK (Cell Signaling Technology Inc., Beverly, MA); c-Myc (Santa Cruz Biotechnology, Inc., Dallas, TX); p-c-Myc (Abcam, Cambridge, MA); GAPDH (GeneTex, Inc., Irvine, CA).

Plasmids and stable cell transfection

The specific shRNA targeting human XIAP and c-Myc was purchased from Open Biosystems (Lafayette, CO). The overexpression of ΔRING and ΔBIR plasmids (21) was described in our previous studies (8, 37). The c-Myc expression plasmid (38) was a gift from Dr. Rosalie Sears (Oregon Health and Science University, Portland, OR). GSK-3β S9A expression plasmid and the plasmid encoding dominant negative ERK2, which contained a K52R mutation of rat ERK2, were created as described previously (39, 40). Cell transfections were performed with PolyJet™ DNA in Vitro Transfection Reagent (SignaGen Laboratories, Rockville, MD) according to the manufacturer’s instructions. For stable transfection, cell cultures were subjected to hygromycin B, G418, or puromycin selection according to the resistance of plasmids, and cells surviving were pooled as stable mass transfectants.

Western blot analysis

Whole-cell extracts were prepared with the cell lysis buffer (10 mm Tris-HCl, pH 7.4, 1% SDS, and 1 mm Na₃VO₄) as described in our previous studies (41). 50 μg of proteins were resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and probed with the indicated primary antibodies together with the alkaline phosphatase–conjugated secondary antibody. Signals were detected by the enhanced chemiluminescence Western blotting system as described in a previous report (22). The images were acquired, and the protein levels were quantified by using the Typhoon FLA 7000 imager (GE Healthcare).

RT-PCR and quantitative RT-PCR

Total RNA was extracted using the TRizol reagent as described in the manufacturer’s instructions (Invitrogen). 5 μg of total RNA was used for first-strand cDNA synthesis with oligo(dT) primer by the SuperScript IV first-strand synthesis system (Invitrogen). Specific primers were used for PCR amplification. The primers used in this study were as follows: human c-myc, forward (5′-AAC ACA CAA CGT CTT GGA GC-3′) and reverse (5′-CCT TAC GCA CAA GAG TTC CG-3′); human gapdh, forward (5′-AGA AGG CTT GGG CTC ATT TG-3′) and reverse (5′-AGG GCC CAT CCA CAG TCT TC-3′); mouse c-myc, forward (5′-TCT CCA CTC ACC AGC ACA ACT ACG-3′) and reverse (5′-ATC TGC TTC CAC CCT TCA AGT-3′); and mouse gapdh, forward (5′-TGC AGT GGG AAA GTG GAG ATT-3′) and reverse (5′-TGG TGC CTC ACC CAC TCA AGT-3′). The quantitative RT-PCR analysis was carried out using the SYBR Green PCR kit (Qiagen, Santa Clarita, CA) and the 7900HT Fast Real-time PCR system (Applied Biosystems, Carlsbad, CA). The ΔΔCT value was used to calculate the relative expression of c-myc mRNA, using gapdh as an endogenous control.

Anchoragel-independent growth assay

Anchoragel-independent growth ability was evaluated in soft agar as described in our previous studies (42). Briefly, 3 ml of
0.5% agar in basal modified Eagle’s medium supplemented with 10% FBS was layered onto each well of 6-well tissue culture plates. 1 ml of 0.35% agar medium with cells (1 × 10⁴ cells) was then layered on top of the 0.5% agar layer. Plates were incubated at 37 °C in 5% CO₂ for 2–3 weeks, and the colonies with more than 32 cells were scored and are presented as colonies/10⁴ cells.

Transwell migration and invasion assay

The migration and invasion kit was purchased from BD Falcon, and the assay was performed according to the manufacturer’s instructions. 30,000 cells were seeded in the upper well with 0.1% serum medium and in the lower part with complete medium, and culture continued for 24 h. Then cells both on the inside and outside of the chamber were fixed with 3.7% formalin for 2 min, washed twice with PBS, transferred to 100% methanol for 20 min, washed twice again, and then finally stained by Giemsa (diluted 1:20 with PBS) at room temperature for 15 min in the dark. They were again washed twice, and then the non-invaded cells were scraped off with a cotton swab (PBS-wetted) four times. The photographs were taken with an Olympus DP71 camera, and the number of cells was calculated by ImageJ software.

Statistical analysis

Student’s t test was used to determine the significance between different groups. p < 0.05 was considered as a significant difference between compared groups.

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