



p63 α protein up-regulates heat shock protein 70 expression via E2F1 transcription factor 1, promoting Wasf3/Wave3/MMP9 signaling and bladder cancer invasion

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Bladder cancer (BC) is the sixth most common cancer in the United States and is the number one cause of death among patients with urinary system malignancies. This makes the identification of invasive regulator(s)/effector(s) as the potential therapeutic targets for managing BC a high priority. p63 is a member of the p53 family of tumor suppressor genes/proteins, plays a role in the differentiation of epithelial tissues, and is believed to function as a tumor suppressor. However, it remains unclear whether and how p63 functions in BC cell invasion after tumorigenesis. Here, we show that p63 α protein levels were much higher in mouse high-invasive BC tissues than in normal tissues. Our results also revealed that p63 α is crucial for heat shock protein 70 (Hsp70) expression and subsequently increases the ability of BC invasion. Mechanistic experiments demonstrated that p63 α can transcriptionally up-regulate Hsp70 expression, thereby promoting BC cell invasion via the Hsp70/Wasf3/Wave3/MMP-9 axis. We further show that E2F transcription factor 1 (E2F1) mediates p63 α overexpression-induced Hsp70 transcription. We also found that p63 α overexpression activates E2F1 transcription, which appears to be stimulated by p63 α together with E2F1. Collectively, our results demonstrate that p63 α is a positive regulator of BC cell invasion after tumorigenesis, providing significant insights into the biological function of p63 α in BC and supporting the notion that p63 α might be a potential target for invasive BC therapy.

Bladder cancer (BC)³ is the sixth most common cancer in the United States and is the number one cause of death among patients with urinary system malignancies (1). The incidence of BC has steadily risen in recent decades. It is estimated that >76,960 Americans will be diagnosed with BC and >16,390 will die of this disease in 2016 (1). Approximately 20–30% of BCs are muscle-invasive, and 50% of these patients die from metastasis within 2 years of diagnosis (2). The 5-year survival rate for metastatic BC is only 5% (2). The remaining 70–80% of BCs are initially diagnosed as non-muscle-invasive, of which ~20% later progress to become muscle-invasive bladder cancer. These progressive BCs have a 43% lower 5-year survival rate than non-muscle invasive cancers (3). The depth of invasion of the bladder wall is closely associated with the clinical treatment of bladder cancers (4). High-grade invasive bladder cancers can rapidly progress to life-threatening metastases, and the 5-year overall survival rate in patients with lymph node-only disease was 20.9% (5). For these reasons the identification of key molecules that are responsible for mediating human BC invasion and metastasis is of tremendous importance for improving the clinical outcome of patients with invasive BCs.

Tumor protein p63, or TP63, is a transcription factor belonging to the p53 family (6). All members of the family have highly conserved domains: a transactivation domain (TA), a DNA-binding domain, and an oligomerization domain (6, 8, 9). The p63 protein has been shown to be important in the development of epithelial tissues. p63-deficient mice have several developmental defects, such as the lack of limbs, teeth, and mammary glands (10). Like other members of the p53 family, the TP63 gene is expressed as multiple isoforms arising by either alternative promoter usage or differential splicing events at the C terminus. In particular, The p63 isoforms containing the transactivation TA domain (TAp63) are capable of efficiently transactivating different p53-responsive genes, such as p21, bax, mdm2, and other unique targets (11), thus largely

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³ The abbreviations used are: BC, bladder cancer; Hsp70, heat shock protein 70; TA, transactivation domain; BBN, *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine; IHC, immunohistochemistry; HSF1, heat shock factor 1; MMP, matrix metalloproteinase; CREB, cAMP-response element-binding protein; IHC-P, immunohistochemistry staining-paraffin; HSF, heat shock factor.

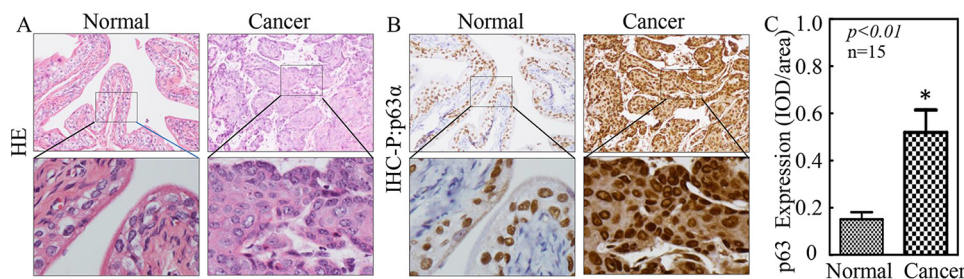


Figure 1. p63 α was up-regulated in mouse BC tissues. A, BBN-induced mouse high invasive BC tissues and normal mouse bladder tissue ($n = 15$) were collected for hematoxylin and eosin (HE) and IHC staining. Hematoxylin and eosin staining was performed, and the representative images of each tissue were captured as described in our previous studies (7). B and C, IHC-P (immunohistochemistry-paraffin staining) was carried out to evaluate p63 α protein expression in mouse BC tissues and normal bladder tissues. The optical density was analyzed as described under "Experimental Procedures." The asterisk (*) indicates a significant increase in comparison with that of normal tissues ($p < 0.05$). IOD/area, integrated optical density per stained area.

mimicking p53 tumor-suppressive activities (11–13). Accordingly, the ectopic expression of TAp63 is able to induce cell cycle arrest and apoptosis (14). Conversely, the ΔN proteins, which lack the canonical TA and are endowed with an alternative activation domain, promote cancer cell survival and tumor progression (15–17). It has been reported that loss of p63 results in spontaneous tumor formation, although the mechanism underlying the tumorigenesis is not yet fully understood (18). p63 α is the longest TA transcript variant of p63 and has been characterized as a tumor suppressor responsible for preventing cancer development (19–23).

Most of the existing studies on p63 α have focused on its role as a tumor suppressor gene (11, 24). Our previous studies demonstrate that the XIAP RING domain mediates miR-4295 expression, inhibiting p63 α protein translation and promoting malignant transformation of bladder epithelial cells (25). However, much less is known about p63 α 's role in invasion and metastasis after tumor formation. In our present studies, we attempted to fill this gap in knowledge and define the function of p63 α in formed human high invasive BC cells. We found that p63 α protein levels were higher in BC tissues than in normal bladder tissues and that p63 α was able to regulate BC invasion via transcriptional regulation of E2F1 expression and subsequent increases in heat shock protein 70 (Hsp70) transcription. We further showed that Hsp70 expression mediated by p63 α was able to promote BC invasion through the Hsp70/Wasf3/Wave3/MMP-9 axis. Given that our current studies together with our published studies show that p63 α inhibits human BC growth, we conclude that p63 α could function as a double-edged sword for its promoting BC invasion accompanied with suppression of human BC growth.

Results

p63 α was up-regulated in mouse bladder cancer tissues

Previous studies have revealed that p63 α can serve as a tumor suppressor (19–23). However, its function in invasion after tumorigenesis remains elusive. *N*-Butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) is a well-characterized bladder carcinogen for its induction of 100% invasive BC in mouse model (26). Consistent with the above, exposure of mice to BBN in drinking water led to 100% (15/15) invasive bladder cancer formation, whereas there were no tumors observed in the control group of 15 mice receiving normal drinking water ($n = 15$) as demon-

strated by pathological hematoxylin and eosin staining (Fig. 1A). The expression level of p63 α in the mouse invasive bladder cancer were further evaluated by using immunohistochemistry (IHC) staining, and the results showed that p63 α protein levels were remarkably increased in BBN-treated mouse bladder cancer tissues in comparison with bladder tissues from mice in control group (Fig. 1, B and C).

p63 α up-regulation promoted BC cell invasion

To elucidate the molecular mechanisms underlying the role of p63 α in BC development, we constructed p63 α overexpression plasmids and stably transfected them into BC cell lines T24, T24T, and UMUC3. The stable transfectants of p63 α and its scramble control were established as shown in Fig. 2, A–C. We then utilized a transwell assay to compare the invasion abilities of the stable transfectants. The invasion abilities of p63 α overexpression cells was significantly increased compared with those of the vector control transfectants (Fig. 2, D–G). It should be noted that based on the manufacturer's instructions for the "transwell invasion assay," migrated cells from transwells without Matrigel were used as the internal control to normalize the invasive cells observed in transwells with Matrigel, which should exclude the effect of cell proliferative factor on cell invasion. Given that our most recent studies indicate that p63 α is able to inhibit EGF-induced anchorage-independent growth of human urothelial cells, an ATP assay was performed to test whether p63 α overexpression inhibited human invasive BC growth. As shown in Fig. 2, H–J, p63 α overexpression did inhibit monolayer growth of all three human high-invasive BC cells tested. These results demonstrate that p63 α serves as an important positive regulator for BC cell invasion.

Hsp70 and Wave3 were up-regulated in p63 α -overexpressed BC cells

To define the mechanism by which p63 α promotes BC cell invasion, we compared the expression levels of key proteins involved in the regulation of BC migration and invasion between scramble vector transfectants and p63 α overexpressed T24, T24T, and UMUC3 cells. As shown in Fig. 3, only Hsp70 and Wave3 were consistently up-regulated in all three human high-invasive BC cell lines with ectopic expression of TAp63 α in comparison with their related scramble vector transfectants, whereas the expression levels of other proteins, including

p63 α regulating Hsp70 for bladder cancer invasion

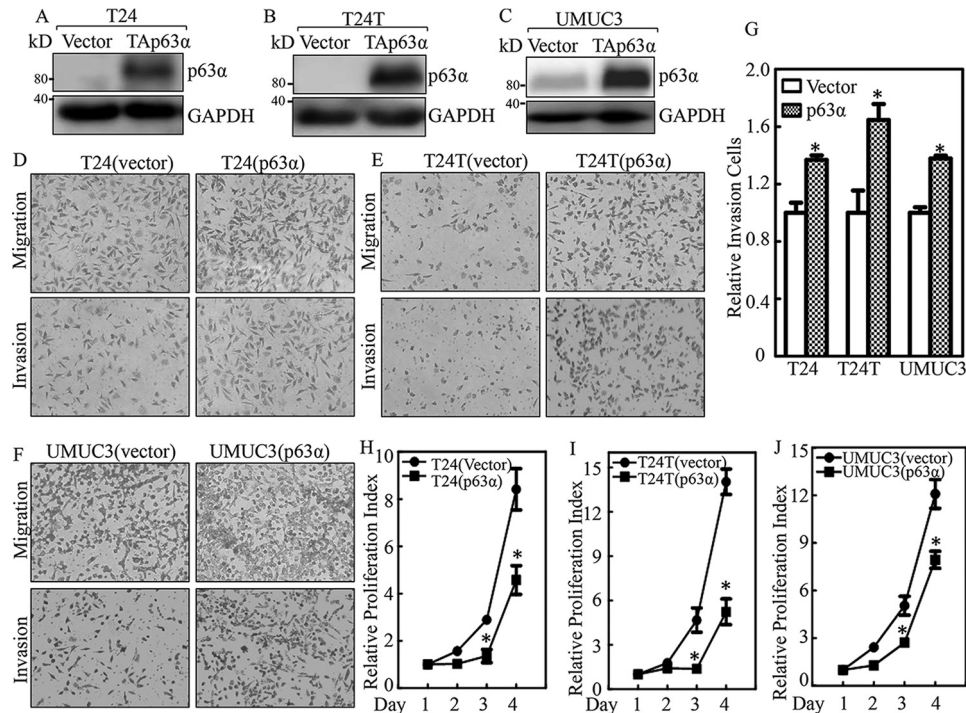


Figure 2. p63 α up-regulation promoted invasion in human high invasive BC cells. A–C, Western blotting was used to evaluate the stable ectopic expression of p63 α in p63 α transfectants as indicated. D–G, the invasion abilities of p63 α stable transfectants and their scramble vector transfectants were evaluated by using BD BioCoat™ Matrigel™ Invasion Chamber. The asterisk (*) indicates a significant difference in invasion ability between p63 α -overexpressed cells and their scramble vector transfectants ($p < 0.05$). The bars are presented as the mean \pm S.D. from three independent experiments. H–J, cell proliferation was determined in the indicated transfectants using ATP assay. The asterisk (*) indicates a significant inhibition as compared with the scramble vector transfectants.

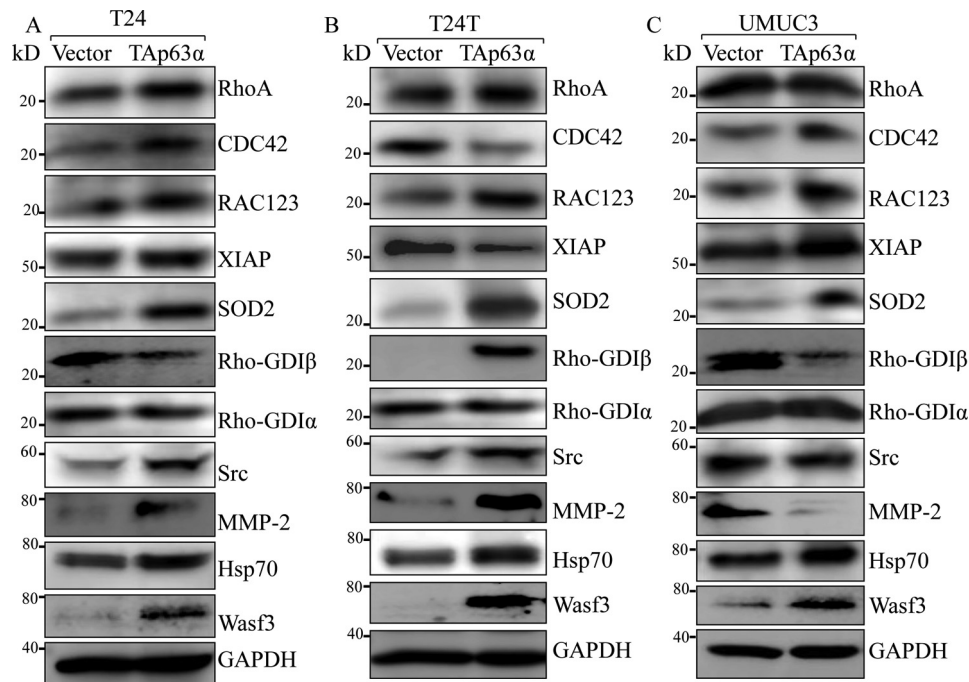


Figure 3. Hsp70 and Wave3 were consistently up-regulated in p63 α ectopic expressed human BC cells. A–C, the indicated cells were seeded into 6-well plates. The cells were extracted upon the cell density reaching 80–90%, and the cell extracts were subjected to Western blot for determination of protein expression as indicated. GAPDH was used as a protein loading control.

RhoA, CDC42, RAC123, XIAP, SOD2, RhoGDI α , RhoGDI β , and SRC did not show consistent alteration in three cell lines (T24, T24T, and UMUC3) after TAp63 overexpression. Our results revealed that Hsp70 and Wave3 may be associated with BC cell invasion.

Hsp70 was crucial for the invasive abilities of BC cells

Hsp70 is an important chaperone protein that plays a key regulator for many cell functions, including cell growth and tumor promotion (27). To test whether Hsp70 contributes to

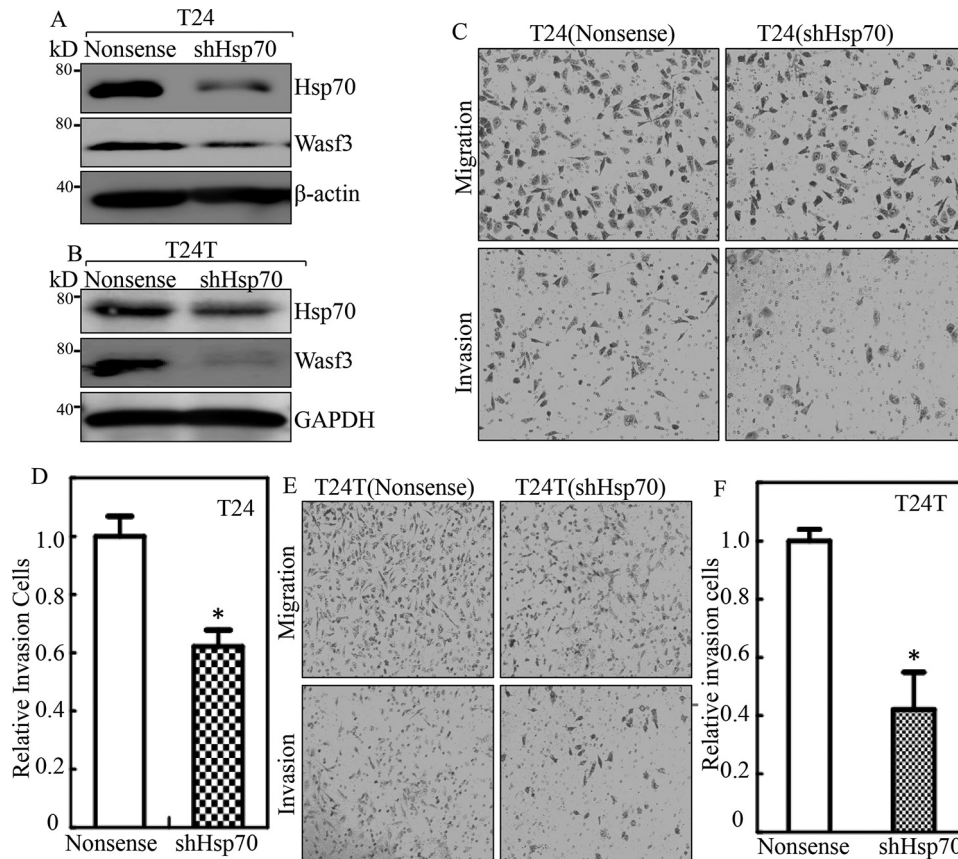


Figure 4. Hsp70 was an important player in the invasion of human BC cells. *A* and *B*, the cell extracts from the indicated cells were subjected to Western blot for determination of levels of Hsp70 and Wasf3. β -Actin or GAPDH was used as a protein loading control. *C–F*, invasion abilities of T24(shHsp70) versus T24(Nonsense) cells and T24T(shHsp70) versus T24T(Nonsense) cells were determined using BD BioCoat™ Matrigel™ Invasion Chamber. The asterisk (*) indicates a significant difference of invasion abilities between T24(shHsp70) versus T24(Nonsense) cells or T24T(shHsp70) and T24T(nonsense) cells ($p < 0.05$). The bars are presented as the mean \pm S.D. from three independent experiments.

increased BC cell invasion, shRNA specifically targeting Hsp70 (shHsp70) was stably transfected into invasive BC cells. The stable transfectant T24(shHsp70), T24T(shHsp70), and their nonsense scramble transfectants were established and identified as shown in Fig. 4, *A* and *B*. The knockdown of Hsp70 led to a decreased invasive ability as compared with their scramble Nonsense transfectants in an invasion assay (Fig. 4, *C–F*). Given that Hsp70 acts as a chaperonin and in turn stabilized Wasf3 protein (28), we tested whether Wasf3 was regulated by Hsp70 in BC cells. The results showed that knockdown of Hsp70 led to a dramatic decrease in Wasf3 protein in T24 and T24T cells as compared with their nonsense scramble transfectants (Fig. 4, *A* and *B*), suggesting that Wasf3 might be a downstream effector of Hsp70 responsible for BC invasion.

Decreased Hsp70 resulted in invasive ability in p63α-overexpressed BC cells, and Wave3 was a downstream effector of Hsp70

To determine whether Hsp70 is required for overexpressed p63α promoting BC cell invasion, we stably transfected shHsp70 into p63α-overexpressed cells T24T(p63α), and the stable transfectants of T24T(p63α/shHsp70-1) and T24T(p63α/shHsp70-2) as well as their related control transfectants T24T(p63α/Nonsense) and T24T(Vector) were established. As shown in Fig. 5*A*, Hsp70 was successfully knocked

down in T24T(p63α/shHsp70-1) and T24T(p63α/shHsp70-2) cells in comparison with T24T(p63α/Nonsense) cells. T24T(p63α/shHsp70-1) and T24T(p63α/shHsp70-2) cells showed a reduction in their invasive abilities when compared with T24T(p63α/Nonsense) cells (Fig. 5, *B* and *C*), suggesting that Hsp70 is an important player in p63α promoting BC invasion. These results indicate that p63α overexpression promotes Hsp70 expression, which plays a critical role in promoting T24T cell invasion. It was noted that knockdown of Hsp70 did not affect BC cell proliferation in p63α-overexpressed T24T cells (Fig. 5*D*). We next investigated how Hsp70 regulated Wasf3 protein. The T24T(p63α/Nonsense) and T24T(p63α/shHsp70) cells were treated with cycloheximide to inhibit new protein synthesis in order to observe the potential regulation of Hsp70 on Wasf3 protein gradation. As shown in Fig. 5, *E* and *F*, Wasf3 protein degradation in T24T(p63α/shHsp70-1) cells was much faster than that in T24T(p63α/Nonsense) cells, suggesting that Hsp70 expression is able to stabilize Wasf3 protein.

p63α promoted Hsp70 transcription by up-regulating E2F1 and Sp1 protein expression

Hsp70 expression is delicately regulated at multiple levels, including transcriptional, post-transcriptional, translational, and post-translational levels (29). Given the above results showing that p63α is important for Hsp70 up-regulation, our

p63 α regulating Hsp70 for bladder cancer invasion

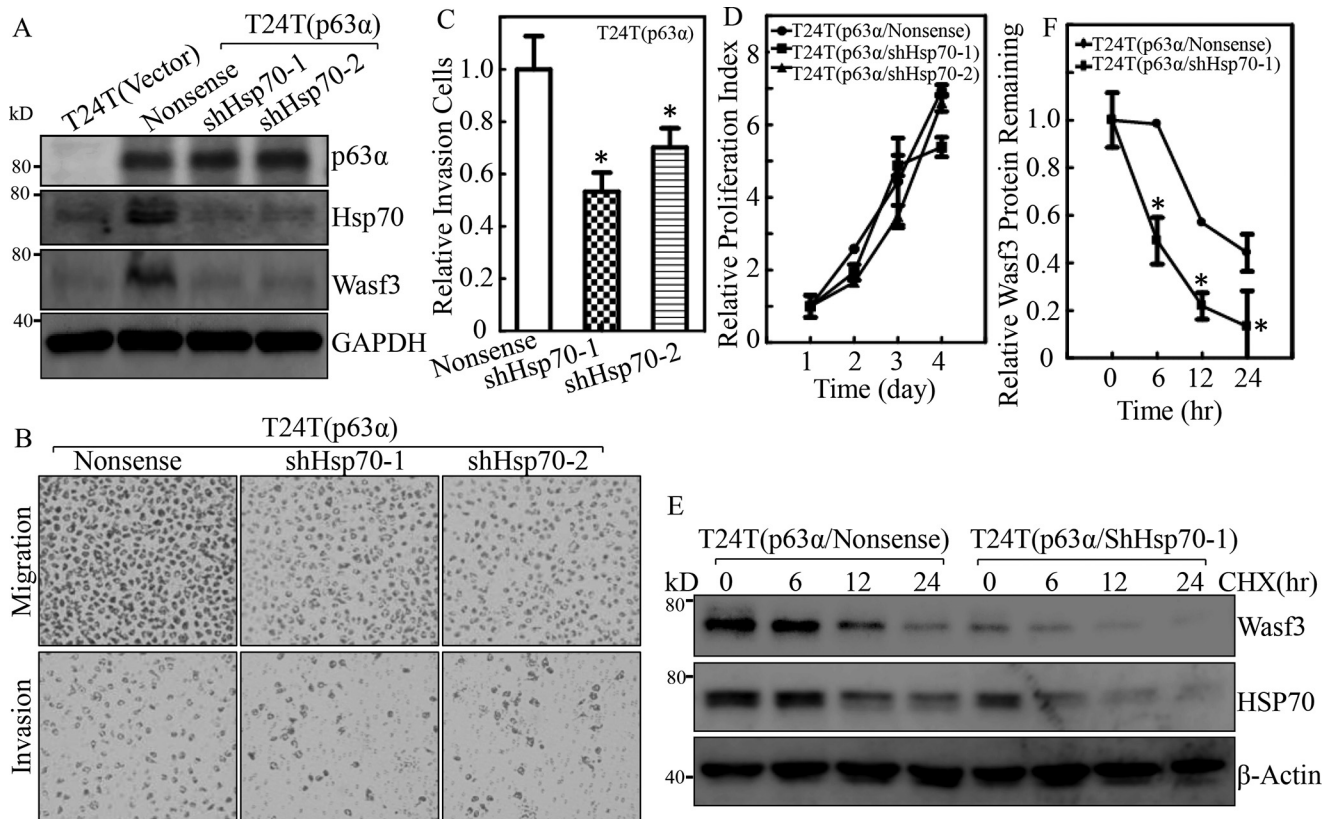


Figure 5. Wave3 was an Hsp70 downstream mediator responsible for p63 α promotion of BC invasion. *A*, T24T(Vector), T24T(p63 α /Nonsense), T24T(p63 α /shHsp70-1), and T24T(p63 α /shHsp70-2) cells were extracted upon the density reaching 80–90%, and the cell extracts were subjected to Western blotting with the specific antibodies as indicated. GAPDH was used as a protein-loading control. *B* and *C*, the invasion abilities of T24T(p63 α /Nonsense), T24T(p63 α /shHsp70-1), and T24T(p63 α /shHsp70-2) cells were subjected to a transwell invasion assay. *B*, the invasion rate was normalized with the insert control according to the manufacturer's instruction, and the results are presented as relative invasion cells. *C*, the asterisk (*) indicates a significant difference of invasion abilities between T24T(p63 α /Nonsense) and T24T(p63 α /shHsp70) cells ($p < 0.05$). *D*, cell proliferation was evaluated by ATG assay. *E*, T24T(p63 α /Nonsense) and T24T(p63 α /shHsp70-1) cells were treated with 50 μ g/ml cycloheximide (CHX) for the indicated time periods, and the cell extracts were subjected to Western blotting to analyze Wasf3 and Hsp70 protein degradation rates. β -Actin was used as a protein loading control. *F*, Wasf3 protein degradations from three independent experiments were analyzed and presented. The asterisk (*) indicates a significant difference between the indicated two transfectants.

subsequent efforts were directed at identifying the mechanisms behind p63 α -mediated Hsp70 up-regulation. Hsp70 mRNA levels were markedly increased in p63 α -overexpressed BC cells as compared with those observed in their control vector transfectants (Fig. 6A). In contrast, the mRNA degradation in p63 α -overexpressed cells was faster than those in control vector transfectants (Fig. 6B). We then evaluated the p63 α regulation of Hsp70 transcription by using an Hsp70 promoter-driven luciferase reporter. We found that Hsp70 promoter activity was significantly increased in p63 α -overexpressed transfectants (Fig. 6C). These results demonstrate that p63 α up-regulates Hsp70 transcription.

To elucidate the mechanisms underlying p63 α up-regulating Hsp70 transcription, TFANSFAC[®] Transcription Factor Binding Sites Software (Biological Database, Wolfenbüttel, Germany) was applied for Bioinformatics analysis of the Hsp70 promoter region. The results indicated that the Hsp70 gene promoter region contains the putative DNA-binding sites for nuclear factor AP-1, Sp1, cAMP-response element-binding protein (CREB)-binding protein (CBP), E2F1, and activating heat shock factor 1 (HSF1; Fig. 6D). We next determined the expression level of these transcription factors in TAp63-overexpressed cells in comparison with the scramble vector trans-

fectants. As shown in Fig. 6, E–G, Sp1 and E2F1 proteins were consistently elevated in TAp63 α -overexpressed BC cells as compared with their scramble vector transfectants, whereas the effect of ectopic expression of TAp63 on expression/phosphorylation of C-Jun, CREB, and HSF1 was not consistent in three type BC cell lines, including T24, T24T, and UMUC3, revealing that Sp1 and E2F1 is modulated by TAp63 α and may participate in hsp70 transcriptional regulation by p63 α .

E2F1 was crucial for Hsp70 transcriptional up-regulation and promotion of BC invasion

Given that both Sp1 and E2F1 protein were increased in ectopic TAp63-expressed BC cells, we determined which of them was more likely to promote Hsp70 transcription. We transfected shRNA-specific targeting human Sp1 (shSp1) (Fig. 7A) or E2F1 overexpressed plasmid into T24T cells (Fig. 7C) in T24T cells. The stable transfectants T24T(Nonsense) and T24T(shSp1) were used to evaluate the effect of Sp1 knock-down on Hsp70 mRNA levels. The results indicated that knock-down of Sp1 did not show any observable effect on Hsp70 mRNA abundance (Fig. 7B), excluding the participating of Sp1 in Hsp70 modulation. The stable transfectants, T24T(vector) and T24T(E2F1), were further employed to evaluate the effects

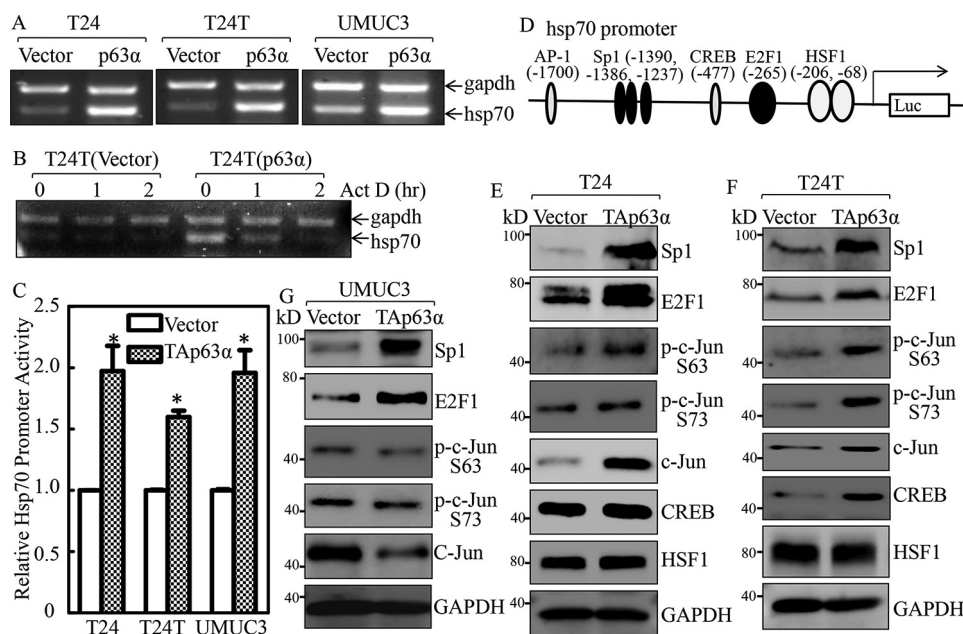


Figure 6. Hsp70 was up-regulated at transcriptional level by p63 α in bladder cancer cells. A, the indicated cells were extracted with TRIzol reagent to isolate total RNA upon the density reaching 80–90%. hsp70 mRNA levels were determined with RT-PCR by using the specific primers. GAPDH was used as an internal control. B, T24T(Vector) and T24T(p63 α) cells were seeded into 6-well plates. After synchronization, T24T(Vector) and T24T(p63 α) cells were treated with actinomycin D (Act D) for the indicated time points, then total RNA was isolated and subjected to RT-PCR analysis to evaluate mRNA levels of hsp70 and GAPDH. C, the indicated cells were transfected with Hsp70 promoter-driven luciferase reporter together with pRL-TK. The transfectants were seeded into 96-well plates and then subjected to determine Hsp70 promoter activity by measuring luciferase activity. pRL-TK was used as an internal control to normalize the transfection efficiency. Each bar indicates the mean \pm S.D. from three replicate assays. The asterisk (*) indicates a significant increase in promoter-driven promoter activity in p63-overexpressed cells in comparison with Vector transfectants ($p < 0.05$). D, potential transcriptional factor-binding sites in the Hsp70 promoter region (–2000 + 1) were analyzed using the TRANSFAC 8.3 engine online. E–G, p63 α stable transfectants as indicated were extracted, and the cell extracts were subjected to Western blotting to determine expression of the indicated proteins. GAPDH was used as a protein loading control.

of E2F1 on Hsp70 expression. As shown in Fig. 7, C and D, Hsp70 mRNA and protein levels were remarkably increased in T24T(E2F1) cells as compared with those in T24T(Vector) cells, revealing the important role of E2F1 in TAp63 promoting Hsp70 mRNA level. To test whether E2F1 regulated Hsp70 mRNA transcription, an Hsp70 promoter-driven luciferase reporter was transfected into T24T(Vector) and T24T(E2F1) cells, and the promoter transcription activities were compared between the two transfectants. As shown in Fig. 7E, Hsp70 promoter-driven luciferase reporter transcription activity was significantly higher in T24T(E2F1) cells than in T24T(Vector) cells, revealing that E2F1 positively regulates Hsp70 mRNA transcription. To test whether E2F1 binds to the Hsp70 promoter region, a chromatin immunoprecipitation (ChIP) assay was carried out in which anti-E2F1 antibodies were used to pull down all E2F1 protein and any DNA with which E2F1 physically interacted. The DNA was then extracted from the precipitated complex, and PCR was performed to detect the presence of the Hsp70 promoter. As shown in Fig. 7F, specific primers of the Hsp70 promoter targeting the E2F1-binding site were used to perform the PCR (top panel of Fig. 7F), whereas the primers of the Hsp70 promoter region that targets the Sp1-binding site was used as a negative control (bottom panel of Fig. 7F). IgG was used as the antibody negative control. The results shown in Fig. 7F strongly indicated that the DNA fragment containing E2F1-binding site, but not Sp1-binding site, was specifically presented in immune-complex pulldown by anti-E2F1 antibodies, revealing the interaction of E2F1 with Hsp70 promoter. In addition, the effect of E2F1 on BC invasion was also evaluated in

E2F1-overexpressed T24T cells. As shown in Fig. 7, G and H, overexpression of E2F1 in T24T cells profoundly promoted invasion of T24T cells. To further determine the role of E2F1 in p63 α promotion of Hsp70 expression and BC invasion, we stably transfected E2F1 shRNA into T24T(p63 α) cells (Fig. 7I). E2F1 knockdown led to an attenuation of Hsp70 expression and invasive abilities of T24T(p63 α) cells (Fig. 7, J and K). Moreover, consistent with p63 α overexpression in BBN-induced mouse-invasive BC, E2F1 was also remarkably overexpressed in same BBN-induced mouse BC tissues (Fig. 7, L and M). These results demonstrate that E2F1 is critical for p63 α promoting Hsp70 transcriptional up-regulation and BC invasion.

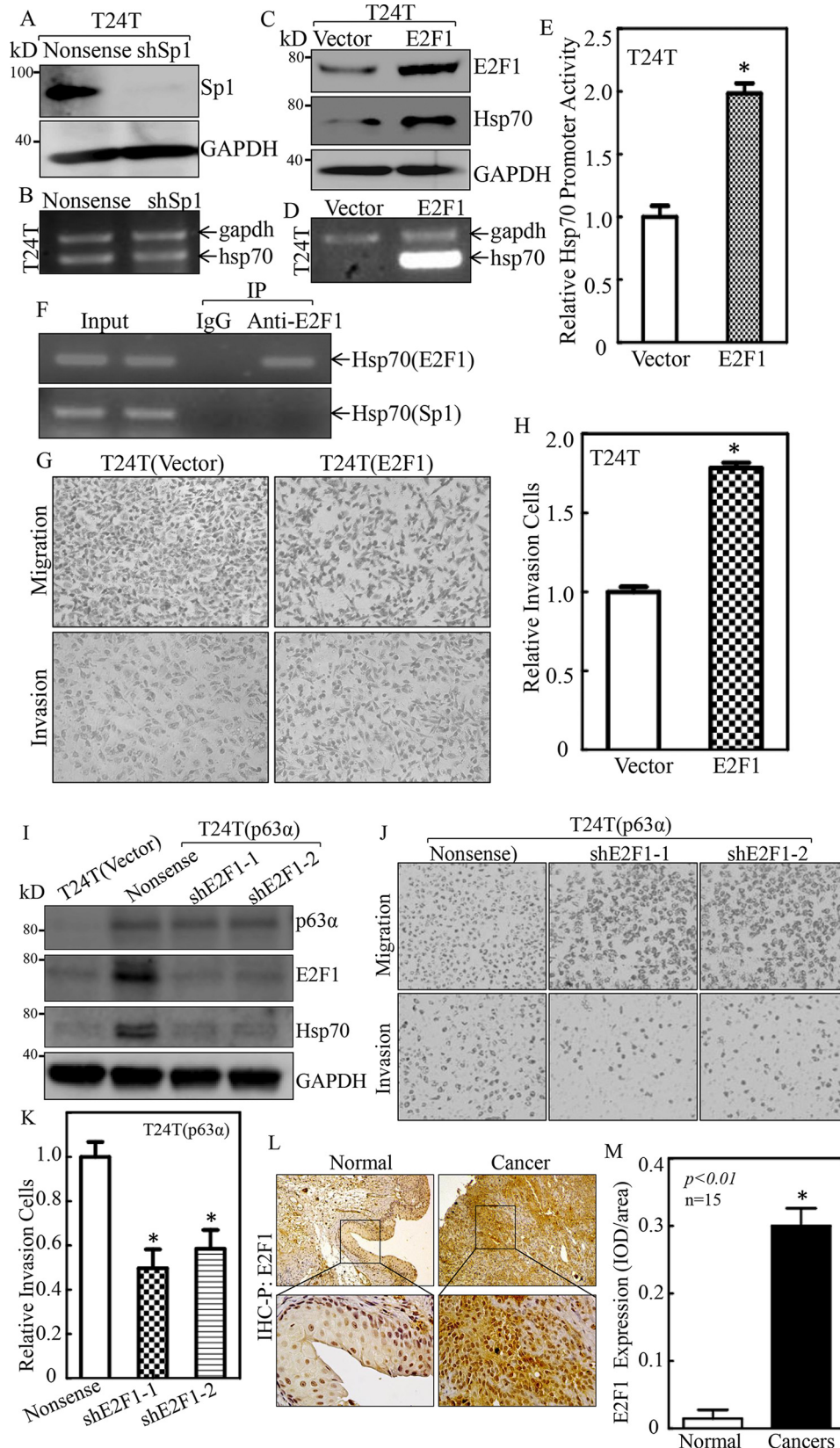
p63 α promoted E2F1 transcription

Given our results showing that E2F1 is important for p63 α up-regulation of Hsp70 expression and BC invasion, our subsequent efforts were directed at elucidating the mechanisms responsible for p63 α up-regulation of E2F1. Therefore, we first examined E2F1 mRNA levels in p63 α -overexpressed cells versus its scramble vector transfectants. The results indicated that E2F1 mRNA levels were significantly elevated in p63 α ectopically expressed cells than those in the scramble vector transfectants in all T24, T24T, and UMUC3 cells (Fig. 8A). The results obtained from evaluation of E2F1 mRNA stability revealed that E2F1 mRNA degradation rate in p63 α transfectants was comparable with their scramble vector transfectants (Fig. 8B). Thus, E2F1 promoter-driven luciferase reporter transcription activities were evaluated in T24T(Vector) and T24T(p63 α) cells. As exhibited in Fig. 8C, E2F1 promoter activity was remarkably

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up-regulated in T24T(p63 α) cells. E2F1 has been reported to be capable of promoting its own transcription (30), and we examined E2F1 promoter activity in T24T(Vector) and T24T(E2F1) cells. As expected, E2F1 promoter activity was markedly

increased in T24T(E2F1) cells in comparison with its scramble vector transfectants (Fig. 8D), strongly suggesting that E2F1 promotes its own mRNA transcription. This notion was greatly supported by the observation of promotion of endogenous



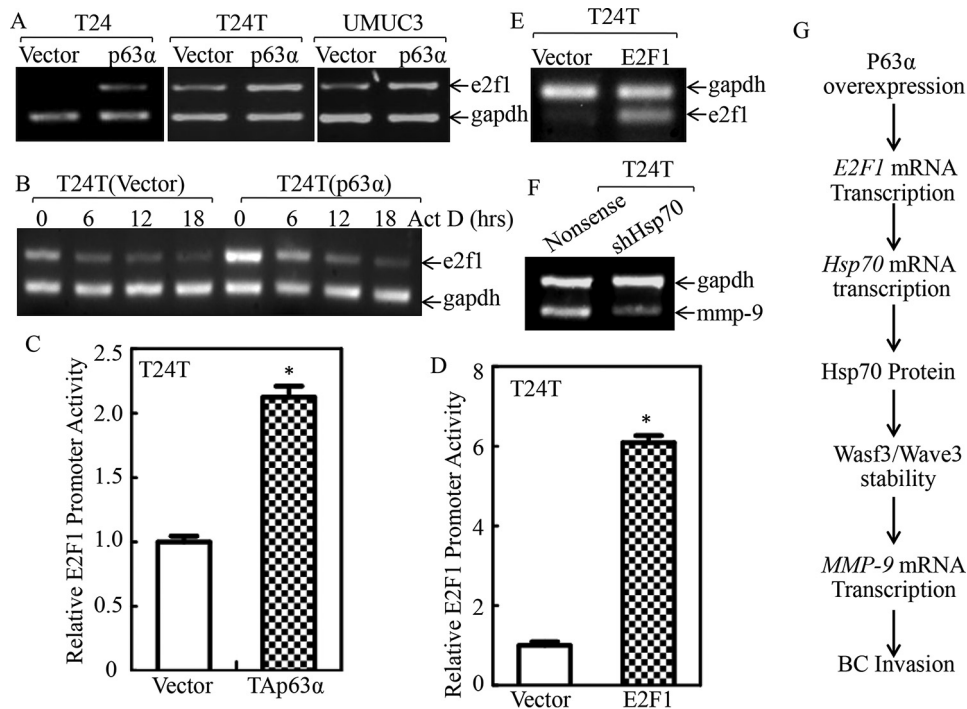


Figure 8. p63 α promoted E2F1 transcription. *A*, the indicated stable transfectants were extracted with TRIzol reagent for total RNA isolation. e2f1 mRNA was determined with RT-PCR using the specific primers. GAPDH was used as an internal control. *B*, the T24T(Vector) and T24T(p63 α) cells were treated with actinomycin D (Act D) for the indicated time points, then total RNA was isolated and subjected to RT-PCR for analysis of e2f1 mRNA degradation. GAPDH was used as a loading control. *C* and *D*, T24T(Vector) versus T24T(p63 α) cells (*C*) and T24T(Vector) versus T24T(E2F1) cells (*D*) were transfected with an E2F1 promoter-driven luciferase reporter together with pRL-TK. The transfectants were seeded into 96-well plates to determine E2F1 promoter transcriptional activity. pRL-TK was used as an internal control to normalize the transfection efficiency. Each bar indicates the mean \pm S.D. from three replicate assays. The asterisk indicates significant increases in E2F1 promoter-driven reporter activity in p63 α -overexpressed cells (*C*) or in E2F1-overexpressed cells (*D*) in comparison with Vector transfectants ($p < 0.05$). *E* and *F*, T24T(Vector) and T24T(E2F1) cells (*E*) or T24T(Nonsense) and T24T(shHsp70) cells (*F*) were extracted for total RNA with TRIzol reagent. RT-PCR was used to determine endogenous e2f1 mRNA expression, whereas GAPDH was used as an internal control. *G*, illustration of proposed molecular mechanisms underlying overexpressed p63 α promoting human BC invasion.

E2F1 mRNA expression in T24T(E2F1) cells (Fig. 8*E*). MMP-9 has been reported to hydrolyze type IV collagen, a key component of the extracellular matrix, and promotes BC invasion (31). Thus, reverse transcription-PCR (RT-PCR) was employed to determine mmp-9 expressions in T24T(shHsp70) and T24T(Nonsense) cells. As shown in Fig. 8*F*, mmp-9 mRNA was remarkably attenuated in T24T(shHsp70) cells, revealing that MMP-9 might act as a downstream effector of p63 α /E2F1/Hsp70 axis responsible for p63 α promoting BC invasion. Taken together, we demonstrated that p63 α overexpression initiated E2F1 mRNA transcription/protein expression through E2F1 self-promoting mechanisms, which also promoted Hsp70 transcription and in turn stabilized Wasf3/Wave3 protein, consequently leading to mmp-9 transcription and BC cell invasion (Fig. 8*G*).

Discussion

Muscular invasive BC is fatal and represents a major therapeutic challenge of this disease. The key to developing future medications is in understanding the mechanisms underlying the affirmative effect on BC invasion. The p63 gene was discovered as the tumor suppressor function along with p73, upon the discovery of the p53 (32). TPp63 contains a transactivation domain (TAD) and can initiate transcription of p53-regulated genes (11). p63 α is the longest TA transcript variant of p63 and has been characterized as a tumor suppressor responsible for preventing cancer development. In this study we found that p63 α was overexpressed in mouse invasive BC tissues as compared with normal bladder tissues and that overexpressed p63 α could promote BC cell invasion. This positive effect on invasion

Figure 7. E2F1 was the transcription factor mediating p63 α promotion of Hsp70 transcription and cell invasion in human BC cells. *A* and *C*, knockdown efficiency of Sp1 or E2F1 ectopic expression in T24T cells was evaluated by Western blotting. GAPDH was used as a protein loading control. *B* and *D*, T24T(Nonsense) versus T24T(shSp1) cells or T24T(Vector) versus T24T(E2F1) cells were extracted for total RNA with TRIzol reagent. RT-PCR was used to determine hsp70 mRNA expression, whereas GAPDH was used as an internal control. *E*, T24T(Vector) and T24T(E2F1) cells were transfected with an hsp70 promoter-driven luciferase reporter together with pRL-TK. The transfectants were used to determine hsp70 promoter activity by measuring luciferase activity. pRL-TK was used as an internal control to normalize the transfection efficiency. Each bar indicates the mean \pm S.D. from three replicate assays. *F*, ChIP assay was carried out as described under "Experimental Procedures," anti-E2F1 specific antibody was used to pull down its E2F1-bound DNA fragments, and primers for E2F1-binding site and negative control Sp1-binding site were used to carry out PCR. *IP*, immunoprecipitation. *G* and *H*, the invasion abilities of T24T(Vector) and T24T(E2F1) cells were determined using a transwell invasion assay. The results are presented as the number of invasive T24T(E2F1) cells relative to invasive T24T(Vector) transfectants (*H*). *I*, the knockdown efficiency of E2F1 in T24T(p63 α) cells was determined by Western blotting. GAPDH was used as a protein loading control. *J* and *K*, the invasion abilities of T24T(p63 α /Nonsense), T24T(p63 α /shE2F1-1), and T24T(p63 α /shE2F1-2) cells were determined using a transwell invasion assay. *L* and *M*, IHC-P was carried out to evaluate E2F1 protein expression in mouse BC tissues and normal bladder tissues. The optical density was analyzed as described under "Experimental Procedures." The asterisk (*) indicates a significant increase in p63 α protein expression in comparison with that observed in normal tissues ($p < 0.05$).

***p63*α regulating *Hsp70* for bladder cancer invasion**

is regulated by E2F1-dependent increases of *Hsp70* mRNA transcription and protein expression. Further studies found that *Hsp70* is crucial for BC cell invasion through the activation of the *Hsp70/Wasf3/Wave3/MMP-9* axis. Our findings that *p63*α up-regulates *Hsp70* mRNA transcription and subsequently activates the *Hsp70/Wasf3/Wave3/MMP-9* axis and promotes BC cell invasion via initiating the transcription factor E2F1 makes a new discovery of *p63*α in promotion of BC invasion, which is distinct from its acting as tumor suppression and further provides new insight into the understanding the double faces of *p63*α in regulation of BC cell growth and progression/invasion in cancer development.

The stress-inducible *Hsp70*, also known as HSPA1A, *Hsp70-1*, *Hsp72*, or *HspA1*, is produced at low or undetectable levels in unstressed, healthy cells (33). Upon a variety of stresses, its expression is rapidly induced through activating HSFs via mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK)- and stress-activated protein kinase (SAPK)-signaling cascades (34, 35). *Hsp70* restores the balance of the cell proteome by normalizing the level of unfolded and denatured proteins. As a molecular chaperone, *Hsp70* is an important regulator in cellular networks, including transcriptional, signaling, membrane, and organelle networks (36). *Hsp70* exerts a dual role in cancer, promoting survival and dissemination of tumor cells while simultaneously contributing to antitumor immunity (37). *Hsp70* overexpression correlates with an aggressive phenotype in several cancer types. Elevated expression of *Hsp70* has been found to correlate with lymph-node metastasis in breast cancer cells (38) and with vascular invasion in gastric cancers (28). In cervical and BC cells, knockdown of *Hsp70* by its shRNA has been shown to suppress invasion and migration (39). The results from Rohde *et al.* (40) also reveal a role of *Hsp70* in regulation of cancer cell adhesion as depletion of *Hsp70* resulted in cell detachment. In the current studies we discovered that *p63*α is able to promote *Hsp70* transcription and BC invasion, which provides a new insight into the understanding of new *Hsp70* function in promotion of high invasive BC progression.

Wasf3, also known as *Wave3*, is a member of the Wiskott-Aldrich family of proteins (41) and is an effector for cancer cell motility, invasion, and metastasis (42–45). *Wasf3* is critical for the regulation of actin cytoskeleton dynamics via activating Arp2/3 complex. This process leads to reorganization of the actin cytoskeleton, mediates cell movement through increasing lamellipodia formation (46), and promotes invasion through activating matrix metalloproteinases (MMPs) (42). Activation of *Wasf3* is achieved to some extent through its phosphorylation by ABL kinase and the resulting lamellipodia formation and invasion in breast cancer cells (43). Compared with lower stage tumors and normal tissue, advanced breast and prostate cancer tumors have increased *WASF3* expression (47, 48). Down-regulation of *WASF3* has been found to inhibit the invasion and metastasis of breast cancer cells, and it has thus been proposed as a metastasis promoter gene (45). *Hsp70* was found in the *WASF3* immuno-complex, and inactivation of *Hsp70* results in destabilization of *WASF3* through proteasome degradation (39); therefore, the influence chaperone proteins such as *Hsp70* show the effect on invasion, at least in part, through

their controlling *Wasf3* protein (39). Our studies showed that *Hsp70* and *Wasf3/Wave3* were up-regulated in *p63*α-overexpressed BC cells, and *WASF3/Wave3* was a downstream effector of *Hsp70* that mediated the increased invasion abilities in *p63*α-overexpressed BC cells. In addition, our results indicated that knockdown of *Hsp70* led to reduction of *MMP-9* mRNA expression, suggesting that *MMP-9* might also be an *Hsp70/WASF3* downstream effector for mediating BC cell invasion.

Transcription factor E2F plays critical roles in cell cycle regulation, apoptosis, and senescence (49, 50). E2F1 and its maximum level of expression is observed in the late G₁/S phase of the cell cycle during which it induces transcription of the genes required for DNA synthesis (50). E2F1 protein is regulated by retinoblastoma protein Rb. When Rb is not in its hyperphosphorylated form, it binds to E2F1 via its pocket domain and inhibits sequence-specific E2F1 DNA-binding, therefore limiting E2F1-mediated transcriptional induction (51). Although E2F1 as a transcription factor induces transcription of many genes, nothing is known about its effect on *Hsp70* expression and potential regulatory effect of *p63* on E2F1 expression. Our studies here indicate that *p63*α can transcriptionally activate E2F1. Interestingly, E2F1 expression could further promote E2F1 transcription in self-regulation manner as well as mediate *Hsp70* mRNA transcription. These studies demonstrate novel mechanisms related to regulation of E2F1 and *Hsp70*.

In summary, our current studies show for the first time that in addition to acting as a tumor suppressor of BC cell growth, *p63*α also promotes BC cell invasion after tumor formation. *p63*α up-regulates *Hsp70* by transcriptionally activating E2F1 and subsequently promoting BC cell invasion via the *WASF3/WAVE3/MMP-9* axis as illustrated in Fig. 8G. This novel mechanism of *p63*α highlights its downstream regulators/effectors as potential biomarkers for diagnosis and/or targets for therapy of invasive and metastatic human BC patients.

Experimental procedures

Reagents, antibodies, and plasmids

Actinomycin D was purchased from Santa Cruz (Dallas, TX). The dual luciferase assay kit was from Promega (Madison, WI). TRIzol reagent and the SuperScript™ First-Strand Synthesis system were from Invitrogen. The shRNA set specifically targeting human *Hsp70* (RHS4533-EG3303) was from Open Biosystems (GE Healthcare). The shRNA-specific knockdown of human *E2F1* was constructed as pSGLV/H1/Green/puro Vector by targeting 5'-TTG ATC ACC ATA ACC ATC T-3' and 5'-TCA GTG AGG TCT CAT AGC G-3'. The *p63*α expression plasmid a gift from Dr. Zhaohui Feng (State University of New Jersey, New Brunswick, NJ). The E2F1 expression plasmid was a gift from Dr. Daniel S. Peeper, The Netherlands Cancer Institute (52). The E2F1 promoter-driven luciferase reporter was obtained from Addgene (Cambridge, MA). The *Hsp70* promoter-driven luciferase reporters were described in our previous study (53). The specific antibodies against *p63*α (GTX102425) and GAPDH (GTX100118) were purchased from Genetex (Irvine, CA); anti-XIAP (610763) antibodies were from BD Biosciences; antibodies specific against RhoA (2117S), CDC42 (2466S), Rac123 (2465S), Src (2109S), *Hsp70* (4872S), *WAVE3*

(2806S), p-c-Jun Ser-63 (2361S), p-c-Jun Ser-73 (3270S), c-Jun (9165S), and CREB (9197S) were from Cell Signaling (Beverly, MA); antibodies specific for RhoGDI α (sc-360), RhoGDI β (sc-11359), Sp1 (sc-59P), E2F1 (sc-193), MMP-2 (sc-13594), and HSF1 (sc-17757) were from Santa Cruz (Dallas, TX).

Cell culture and transfection

T24 and T24T cells were described in our previous publications (31) and cultured in DMEM:F-12 (1:1) with 5% FBS (Atlanta Biologicals, Flowery Branch, GA). UMUC3 was described in our previous studies (54, 55) and cultured in DMEM with 10% FBS (Atlanta, Flowery Branch, GA). Cell transfections were performed with PolyJetTM DNA (SL10068) in Vitro Transfection Reagent (SigmaGen Laboratories, Rockville, MD) according to the manufacturer's instructions. For stable transfection, T24, T24T, and UMUC3 cells were subjected to selection with hygromycin B (200 μ g/ml) (31282-4-9, Goldbio, Ashby Rd., St. Louis, MO), G418 (1000 μ g/ml) (sc-29065, Dallas, TX), or puromycin (0.3 μ g/ml) (sc-205821, Dallas, TX) depending on the antibiotic resistance of different plasmids transfected. The cells surviving from the antibiotics selection were pooled as mass stable transfectants.

Luciferase reporter assay

Bladder cancer cells were co-transfected with either the Hsp70 or E2F1 promoter-driven luciferase reporter constructs together with the Renilla luciferase vector pRL-TK (Promega). The luciferase activities were determined by using the dual-luciferase assay kit (Promega) together with a luminometer (Lumat LB9507, Berthold Tech., Bad Wildbad, Germany). The firefly luciferase signal was normalized to the Renilla luciferase signal for each individual analysis to eliminate the variations of transfection efficiencies as previously described (56).

N-butyl-N-(4-hydroxybutyl) nitrosamine-induced high invasive mouse BC and immunohistochemistry staining-paraffin (IHC-P)

All animal procedures were approved by the Committee on Animal Resources of the New York University School of Medicine and in accordance with NIH guidelines. C57BL/6 male mice ($n = 15$ /group) at ages of 3–4 weeks were supplied *ad libitum* with tap water containing 0.05% BBN (B0938, TCI America, Portland, OR) in opaque bottles for 23 weeks, whereas negative control mice received regular tap water. The drinking water was prepared freshly twice a week, and consumption was recorded to estimate BBN intake. Mice were sacrificed at 23 weeks after the experiment began, and bladders were harvested and preserved in paraffin for pathological analysis and immunohistochemistry staining (IHC).

Bladder tissues obtained from the sacrificed mice specimens were formalin-fixed and paraffin-embedded as described in our previous studies (26). Immunohistochemistry staining (IHC) was performed to evaluate p63 α expression between BBN-induced invasive bladder cancer tissues and negative control bladder tissues. IHC was performed using antibodies specific against p63 α (Genetex) together with the IHC kit following the protocol described in our previous studies (57). The resultant immunostaining images were captured using the AxioVision

Rel.4.6 computerized image analysis system (Carl Zeiss, Oberkochen, Germany). p63 α protein expression levels were analyzed by calculating the integrated optical density per stained area (IOD/area) using Image-Pro Plus version 6.0 (Media Cybernetics). The IHC-stained sections were evaluated at 400-fold magnifications. At least five representative staining fields of each section were analyzed to calculate the optical density based on typical photographs that had been captured.

RT-PCR

Total RNA was extracted with TRIzol reagent, and 5 μ g of total RNA was used for first-strand cDNA synthesis with oligo(dT) primer by SuperScriptTM First-Strand Synthesis system (Invitrogen). Specific primer pairs were designed for amplifying human hsp70 (forward, 5'-CAA CAC GGC AAG GTG GAG TCA-3'; reverse, 5'-TCA GCC GCT TCG CGT CAA ACA-3'), e2f1 (forward, 5'-GAG GTG CTG AAG GTG CAG AA-3'; reverse, 5'-GTT TGC TCT TAA GGG AGA TCT G-3'), endogenous e2f1 (forward, 5'-AGT TCA TCA GCC TTT CCC CAC C-3'; reverse, 5'-GAC AAG GTG AGC ATC TCT GGA AAC-3'), and GAPDH (forward, 5'-AGA AGG CTG GGG CTC ATT TG-3'; reverse, 5'-AGG GGC CAT CCA CAG TCT TC-3'). The PCR products were separated onto 2% agarose gels and stained with ethidium bromide, and the images were scanned with a UV light as described previously (58).

Immunoblotting assay

Whole cells were washed with ice-cold PBS, and then extracted with cell lysis buffer (10 mM, pH 7.4, Tris-HCl, 1% SDS, 1 mM Na₃VO₄, and proteasome inhibitor). The protein concentration was determined using Nano Drop 2000 (Thermo Scientific, Holtsville, NY). The cell extracts were subjected to Western blotting with each of the antibodies as indicated. The protein bands specifically bound to the primary antibodies were detected using an alkaline phosphatase-linked secondary antibody and ECF (enhanced chemifluorescence) Western blotting analysis system (Amersham Biosciences) as previously described (59). The results shown are representative one from at least three independent experiments.

Cell invasion assay

The invasion kit was purchased from BD Falcon (354480) (Franklin Lakes, NJ). The invasion assay was performed according to the manufacturer's instructions in normal cell culture serum. Upon incubation of the transwell, the cells on both 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 finally stained by Giemsa (1:20 diluted with PBS) at room temperature for 15 min in the dark. After staining, the cells were washed twice again with PBS, and the non-invaded cells were scraped off with a cotton swab (PBS-wetted) four times. The cell invasion ability was calculated by invasion normalized to migrated cells. The migration was determined in transwells without Matrigel. The images were captured under an Olympus DP71, and the number of cells was calculated by the software ImageJ as described in previous papers (26, 31).

p63 α regulating Hsp70 for bladder cancer invasion

ChIP assay

The EZ-ChIP kit (Millipore Technologies) was used to carry out the ChIP assay according to the manufacturer's instructions and as described previously (60). Briefly, T24T cells were treated with 1% formaldehyde for 10 min at room temperature. Cells were then pelleted, resuspended in lysis buffer, and sonicated to generate 200–400 bp of chromatin DNA fragments. After centrifugation ($13,000 \times g$ at 4 °C) for 10 min, the supernatants were incubated with an anti-E2F1 antibody or the control rabbit IgG at 4 °C overnight. The immune complex was captured with Protein G-agarose-saturated beads with salmon sperm DNA and then eluted with elution buffer. The reverse cross-linking of protein-DNA complexes to free DNA was conducted by incubating at 65 °C overnight. The DNA was extracted and subjected to PCR analysis. To specifically amplify the region containing the E2F1-binding sites on the human Hsp70 promoter, PCR was performed with the pair of primers specific for E2F1 bind site, 5'-TCC ATT GTA ACG TGG CCG G-3' and 5'-CTA ATT GAC AGG AAG GGT CGG C-3', whereas the primers specific for the Sp1-binding site were 5'-TTT CTG GAG CCA ATA ACT GA-3' and 5'-TGT TAG CCA GGA TGG TAG CC-3', used as negative control. PCR products were separated on 2% agarose gels and stained with ethidium bromide. The gels were scanned under UV light as described previously.

Statistical analysis

Student's *t* test was used to determine the significance between treated and untreated groups. The results are expressed as the mean \pm S.D. from at least three independent experiments. $p < 0.05$ was considered a significant difference between compared groups (61).

Author contributions—H. H., X. R. W., and C. H. designed the studies. H. J., Q. X., and A. W. drafted the manuscript, J. X. carried out the IHC assay, H. J. and X. G. conducted the real-time PCR assays, H. J. and J. X. performed the Western blotting assays and luciferase reporter assays. H. J., H. H., and C. H. performed the statistical analysis, J. L. helped to acquire the experimental data, and J. Z. detected the cells' biological function. All authors read and approved the final manuscript.

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