The Regulatory Mechanism of Extracellular Hsp90α on Matrix Metalloproteinase-2 Processing and Tumor Angiogenesis

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Heat shock protein 90α (Hsp90α) is a ubiquitously expressed molecular chaperone that is essential for eukaryotic homeostasis. Hsp90α can also be secreted extracellularly, where it has been shown to be involved in tumor metastasis. Extracellular Hsp90α interacts with and promotes the proteolytic activity of matrix metalloproteinase-2 (MMP-2). However, the regulatory mechanism of Hsp90α on MMP-2 activity is still unknown. Here we show that Hsp90α stabilizes MMP-2 and protects it from degradation in tumor cells. Further investigation reveals that this stabilization effect is isoform-specific, ATP-independent, and mediated by the interaction between the Hsp90α middle domain and the MMP-2 C-terminal hemopexin domain. Moreover, this mechanism also applies to endothelial cells that secrete more Hsp90α in their proliferating status. Furthermore, endothelial cell transmigration, Matrigel plug, and tumor angiogenesis assays demonstrate that extracellular Hsp90α promotes angiogenesis in an MMP-2-dependent manner. In this study, we provide new insights into the molecular mechanism of how Hsp90α regulates its extracellular client proteins and also reveals for the first time the function of extracellular Hsp90α in promoting tumor angiogenesis.

Heat shock protein 90 (Hsp90)2 is an ATP-dependent molecular chaperone that is ubiquitously expressed and essential for cell viability (1). Unlike other types of chaperones, Hsp90 is not required for the biogenesis of most polypeptides but instead functions in the maintenance of the active state of several conformationally labile signaling proteins (2–4). Many of the Hsp90 client proteins are mutated, chimeric, or overexpressed oncogenic proteins (3). Therefore, the chaperoning function of Hsp90 is subverted to a biochemical buffer for genetic lesions in tumor cells, facilitating the malignant transformations of the cells (3). Hsp90 has emerged as a promising target for cancer therapy (5).

There are two isoforms of Hsp90 in the cytosol, referred to as Hsp90α and Hsp90β (6). Intriguingly, the Hsp90α isoform also exists extracellularly (7). Recent studies indicate that extracellular Hsp90α is significantly correlated with tumor invasiveness and metastasis (8), and the antibody or impermeable inhibitor of Hsp90α can suppress tumor metastasis efficiently in mouse models (9–11). Furthermore, Hsp90α can be detected in the blood of cancer patients, and the level of Hsp90α is positively associated with tumor malignancy (9). In addition to tumor cells, extracellular Hsp90α has also been identified in neuron cells, dermal fibroblasts, keratinocyte, macrophages, and epithelial cells and participates in neuronal cell migration, wound healing, and viral and bacteria infections (7).

Accumulating evidence indicates that extracellular Hsp90α plays important roles in both physiological and pathological processes, especially in tumor progression (7). However, the molecular mechanism of how extracellular Hsp90α functions is still largely unknown (12). Eustace et al. (8) reported that extracellular Hsp90α can interact with matrix metalloproteinase 2 (MMP-2) and that the impermeable inhibitor of Hsp90α (immobilized geldanamycin) inhibits MMP-2 proteolytic activity, but the regulatory molecular mechanism behind this phenomenon is still a mystery.

In the present study, to further elucidate the molecular mechanism of extracellular Hsp90α function, we have investigated the regulatory mechanism of extracellular Hsp90α on MMP-2 activity. We reveal that extracellular Hsp90α stabilizes MMP-2 and protects it from processing and subsequent inactivation in tumor cells. The regulatory function of Hsp90α on MMP-2 processing is isoform-specific and ATP-independent. The interaction of Hsp90α and MMP-2 is mediated by the middle domain of Hsp90α and the C-terminal hemopexin domain of MMP-2. Moreover, we further confirm this mechanism in endothelial cells, which can secrete increasing amounts of Hsp90α upon the treatment of VEGF. The effects of recombinant human Hsp90α (rHsp90α) and the Hsp90α antibody on angiogenesis in vitro and in vivo were also examined. The result shows that rHsp90α promotes whereas the antibody of Hsp90α suppresses angiogenesis in an MMP-2-dependent manner, suggesting that extracellular
Hsp90α is a potential therapeutic target for not only tumor metastasis but also tumor angiogenesis.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Transfectants**—Human breast cancer cell lines MDA-MB-231 and MCF-7 and mouse melanoma cell line B16/F10 were from the American Type Culture Collection. HMEC is a human dermal microvascular endothelial cell line (Sciencell) transfected with SV40 large T antigen (13). Human umbilical venous endothelial cells (HUVECs) were isolated from human umbilical vein (14). The stable cell line overexpressing MMP-2 was screened by G418 (200 μg/ml) from MCF-7 cells transfected with pcDNA3.1–MMP-2.

**Antibodies**—Anti-Myc, anti-His, and anti-CD31 antibody (PECAM-1, M-20) was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-MMP-2 antibody (Ab-7) for Western blotting was from Calbiochem (Darmstadt, Germany). Anti-Hsp90α antibody (9D2) was from Stressgen Bioreagents (Victoria, Canada). Anti-Hsp90β antibody (H90–10) was from Abcam (Cambridge, UK). Anti-FLAG antibody was from Sigma. Monoclonal antibody against Hsp90α (Hsp90α mAb) and Hsp90β (Hsp90β mAb) for endothelial cell transmigration, tube formation, and tumor growth assay was prepared by our laboratory. The specificity and efficiency were confirmed by ELISA.

**MMP-2 Processing Assay in Vitro**—This assay was performed according to the previous report (15). Purified rMMP-2 was incubated with the indicated proteins with or without ATP (2 mM) at 37 °C for different periods. The incubation buffer contains 40 mM HEPES, 10 mM MgCl₂, 2 mM CaCl₂, 5 mM KCl, 2 mM p-aminophenylmercuric acetate, pH 7.4. The processing was terminated by electrophoresis sample buffer.

**Endothelial Cell Transmigration Assay**—This assay was performed using 6.5-mm millipore inserts (8-μm pore size; Millipore) coated with Matrigel (50 μg/insert) (Vigorous, Beijing, China) on the upper side of the filter membranes. 2 × 10⁵ HMECs/insert were inoculated. 0.5% FBS was added in the lower side of the filter membranes. 2 μg/ml of VEGF-A165 (50 ng/ml) (PeproTech, Rocky Hill, NJ), lentivirus with vector expressing MMP-2 shRNA or empty vector (5 × 10⁶ TU) (GenePharma, Shanghai, China), and recombinant Hsp90α (rHsp90α), recombinant Hsp90β (rHsp90β), nonimmune mouse IgG, Hsp90α mAb, or Hsp90β mAb (50 μg/ml) was injected subcutaneously into the nude mice. After 7 days, the plug was removed, and tube formation was examined by immunofluorescence using anti-CD31 antibody. The target sequence of MMP-2 shRNA is AAGAACCAGATCATAACGAG.

**Tumor Growth Assay**—B16/F10 cells (1 × 10⁶, 100 μl) were subcutaneously inoculated in the back of nude mice (6–8 weeks old). Nonimmune mouse IgG (10 mg/kg), Hsp90α mAb (5 or 10 mg/kg, according to the previous report (10)), or Hsp90β mAb (5 or 10 mg/kg) was injected intraperitoneally every other day from the day after implantation. The experiment was performed with six mice in each group. After 25 days treatment, the mice were sacrificed, and the tumors were excised, weighed, fixed, and applied to immunofluorescence.

**RESULTS**

**Hsp90α Prevents the Processing and Inactivation of MMP-2**—To investigate the regulatory mechanism of Hsp90α on MMP-2 activity, an MCF-7 stable cell line that overexpresses human MMP-2 with C-terminal tandem Myc and His tags was established (Fig. 1A). MMP-2 expression in the conditioned medium (CM) of this cell line treated with rHsp90α or its antibody was analyzed. As expected, activated MMP-2 was increased upon the treatment with increasing amounts of rHsp90α (Fig. 1, B and C), which is consistent with the previous report (8). Intriguingly, the amount of ProMMP-2 was also increased, whereas some overprocessed fragments of MMP-2 (~30 kDa), which were detected by Western blotting but not zymography (Fig. 1, B and C), were decreased in this process (Fig. 1B). Furthermore, the antibody of Hsp90α exhibited the opposite effect on MMP-2 processing (Fig. 1, D and E), whereas rHsp90β and anti-Hsp90β antibody showed no such effect (supplemental Fig. S1). These results indicate that Hsp90α can modulate the activity of MMP-2 via interfering with the processing or degradation of MMP-2 at a specific cleavage site. In addition, the densitometry reading result of the MMP-2 processing upon the treatment of rHsp90α or anti-Hsp90α antibody also showed that Hsp90α was mainly involved in the inactivation processing but not the activation of MMP-2 (Fig. 1, F and G).

We next sequenced the major processed fragment (~30 kDa) observed above and identified its N terminus as YLGAS using Image-Pro Plus.

**In Vivo Matrigel Plug Assay**—400 μl of Matrigel containing VEGF-A165 (50 ng/ml) (PeproTech, Rocky Hill, NJ), lentivirus with vector expressing MMP-2 shRNA or empty vector (5 × 10⁶ TU) (GenePharma, Shanghai, China), and recombinant Hsp90α (rHsp90α), recombinant Hsp90β (rHsp90β), nonimmune mouse IgG, Hsp90α mAb, or Hsp90β mAb (50 μg/ml) was injected subcutaneously into the nude mice. After 7 days, the plug was removed, and tube formation was examined by immunofluorescence using anti-CD31 antibody. The target sequence of MMP-2 shRNA is AAGAACCAGATCATAACGAG.
minal tags, the molecular mass of the fragment is increased to 27.4 kDa, which is consistent with its migratory position on the SDS-PAGE (Fig. 1, B and D). According to the previous report, this fragment is a stable and inactive product of MMP-2 autocatalytic degradation (15, 16). The cleavage at Glu443–Leu444 initiates the MMP-2 inactivation, which is completed later by cleavage at the zinc-binding domain (15). Based on our results and aforementioned literature, we propose that Hsp90α/H9251 can stabilize MMP-2 and protect it from autocatalytic cleavage at Glu443–Leu444, which subsequently leads to the complete inactivation of MMP-2.

**Hsp90α Stabilizes MMP-2 in an ATP-independent and Isoform-specific Manner**—To affirm the above hypothesis, we prepared the purified rHsp90α, rHsp90β (supplemental Fig. S3A), and rProMMP-2 (supplemental Fig. S3B) and investigated the influence of these chaperones on MMP-2 autocatalytic processing in an *in vitro* noncell system. Because Hsp90 is an ATP-dependent molecular chaperone in the cytosol (17, 18), we first examined whether the stabilization effect of Hsp90α on MMP-2 is ATP-dependent. Purified rProMMP-2 was mixed with PBS, equal molar of Hsp90α or Hsp90β with or without ATP, and then incubated at 37 °C for 3 h. The autocatalytic processing products of ProMMP-2 were assayed by Western blotting. It was found that Hsp90α but not Hsp90β can protect MMP-2 from autocatalytic processing and inactivation and that ATP exhibited no effect on the stabilization activity of both Hsp90α and Hsp90β (supplemental Fig. S3C), demonstrating that the stabilization of MMP-2 mediated by Hsp90α is ATP-independent.

Next the effect of Hsp90α and Hsp90β on MMP-2 processing was compared at different incubation times. Without the protection of other factors, only 20% of pro- or active MMP-2 remained after incubation at 37 °C for 1 h (Fig. 2, A, top left, and B), whereas with equal molar amounts of rHsp90α, >90% MMP-2 were in their pro- or activated forms after 1 h of incubation, and even after 20 h of incubation, more than 30% MMP-2 was still active (Fig. 2, A, top right, and B). This result is indicative that Hsp90α has the ability to remarkably
stabilize MMP-2. Similar to the result shown in supplemental Fig. S3C, here Hsp90β also showed no obvious effect on the stabilization of MMP-2 (Fig. 2, A, bottom, and B).

To further confirm this result, the effects of Hsp90α and Hsp90β on MMP-2 stabilization were compared in a concentration gradient. The result showed that after incubation at 37 °C for 1 h (without Hsp90α or Hsp90β), 80% of pro-active or active MMP-2 was processed into small inactive fragments, whereas equal molar amounts of Hsp90α can inhibit the inactivation processing of MMP-2 completely (Fig. 2, C, top, and D). As for Hsp90β, even after increasing its concentration to 40 μM, which is 8-fold of the concentration of MMP-2, the inactivation processing of MMP-2 could not be inhibited completely (Fig. 2, C, bottom, and D). Based on the above results, we conclude that Hsp90α can stabilize MMP-2 directly and specifically.

Because Hsp90α and Hsp90β are closely related isoforms of Hsp90, which are 86% identical and 93% similar in their amino acid sequences (19, 20), it seems contradictory that Hsp90β shows no effect on the stabilization of MMP-2. To investigate this mystery, the status of Hsp90α and Hsp90β during incubation with MMP-2 was explored. Interestingly, Hsp90α was found to be gradually and slowly degraded by equal molar amounts of MMP-2 within 20 h (supplemental Fig. S4A), whereas Hsp90β was rapidly degraded within 1 h and was found to be much more unstable than Hsp90α (supplemental Fig. S4B). This result provides an explanation for the different behaviors of Hsp90α and Hsp90β in the stabilization of MMP-2, which is determined not only by the interaction with MMP-2 but also by the individual stabilities of each chaperone.
The Hemopexin Domain Is Essential for the Interaction of MMP-2 with Hsp90α—To investigate the molecular mechanism of the stabilization of MMP-2 by Hsp90α, the binding region of MMP-2 to Hsp90α was mapped. The different functional domains of MMP-2 are shown in the top panel of Fig. 3A. Mutants ΔPEX (full-length MMP-2 lacking the hemopexin domain) and PEX (hemopexin domain with the signal peptide for secretion) were constructed, and their interactions with Hsp90α were examined by co-immunoprecipitation (Co-IP). Hsp90α was co-precipitated with both the MMP-2 full length and PEX but not ΔPEX (Fig. 3A, bottom), demonstrating that MMP-2 binds to Hsp90α via the hemopexin domain.

The interaction of Hsp90α with the hemopexin domain was further confirmed by a competitive Co-IP assay. Increasing amounts of GST-fused recombinant hemopexin domain (GST-PEX, 0, 1, 10 μg/ml) were added to the concentrated CM of MCF-7 stable cell line overexpressing MMP-2 (Fig. 3B, top left). The Co-IP of Hsp90α and MMP-2 was blocked by GST-PEX in a dose-dependent manner (Fig. 3B, top right and bottom left), whereas the
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Co-IP of Hsp90α and GST-PEX was increased along with the concentration gradient of GST-PEX (Fig. 3B, top right and bottom right). In sum, these results consistently demonstrate that the hemopexin domain is essential for the interaction of Hsp90α and MMP-2.

Hsp90α binds to MMP-2 via its middle domain—Next the binding site of Hsp90α to MMP-2 was mapped. We constructed FLAG-tagged truncation mutants of Hsp90α according to its functional domains, the N-terminal, middle, and C-terminal domains, which are schematically shown in the top panel of Fig. 3C. Then we transfected these truncations to MCF-7 cells and used anti-FLAG antibody to perform Co-IP. The precipitated proteins were detected by anti-MMP-2 antibody. The result showed that MMP-2 was co-precipitated with Hsp90α full length, middle, N-terminal/middle, and middle/C-terminal domains but not the N-terminal or C-terminal domain, demonstrating that Hsp90α interacts with MMP-2 via its middle domain (Fig. 3C, bottom left). Intriguingly, the MMP-2 proteins co-precipitated with Hsp90α were mainly in the activated form (Fig. 3C, bottom left), implicating the specific stabilization effect of Hsp90α on the activated MMP-2.

It is reported that the middle domain of Hsp90α is responsible for the binding of several Hsp90α substrate proteins and is considered to discriminate different substrate types and to regulate the chaperone machinery for proper substrate activation (21). According to the reported mutations, which were shown to be important for the binding of these substrates (21, 22), we constructed point mutations of Hsp90α (Fig. 3C, top) and screened the binding sites of Hsp90α to MMP-2. Strikingly, we identified that three clusters of mutations, F349A/L351A/F352A, R400A/E401K, and V368A/F369A can completely abrogate the interaction of Hsp90α to MMP-2 (Fig. 3C, bottom right), whereas another mutation, W320A, which disrupts the binding of PKB/AKT to Hsp90α (23), showed no such effect (Fig. 3C, bottom right).

Subsequently, we prepared the recombinant proteins of Hsp90α mutants F349A/L351A/F352A and R400A/E401K and then compared their abilities on MMP-2 stabilization (Fig. 3D), demonstrating that the protection of Hsp90α on MMP-2 requires physical interaction, and this further confirms that Hsp90α can specifically stabilize MMP-2.

Hsp90α promotes the transmigration and tube formation of endothelial cells—Because we demonstrated that Hsp90α stabilizes MMP-2 and protects it from inactivation by binding to the hemopexin domain, we further explored the biological relevance of this mechanism. In addition to the cancer cells, endothelial cells also express a high level of MMP-2 (24), which is an important positive regulator of angiogenesis (12); we thus considered whether Hsp90α can be secreted by endothelial cells, which would subsequently be involved in the modulation of angiogenesis. To address this question, the secretion of Hsp90α from two kinds of endothelial cells, HMECs and HUVECs, was examined. Without any stimulation, both HMECs and HUVECs secrete a low level of Hsp90α compared with tumor cells (Fig. 4A), whereas when endothelial cells were treated with increasing amounts of vascular endothelial growth factor (VEGF-A165), which can stimulate the proliferation and induce the angiogenic responses of endothelial cells, the secretion of Hsp90α from both HMECs and HUVECs was increased remarkably (Fig. 4, B and C), suggesting that the secretion of Hsp90α is correlated with the angiogenic status of endothelial cells.

Subsequently, we examined the influence of rHsp90α and Hsp90α mAb on MMP-2 processing and angiogenesis using endothelial cells. rHsp90β and Hsp90β mAb were taken as the control proteins. As expected, rHsp90α inhibited whereas Hsp90α mAb promoted the inactivation processing of MMP-2 in HMECs (Fig. 5A). rHsp90β and Hsp90β mAb exhibited no obvious effect on MMP-2 processing (Fig. 5A). Coincidently, the transmigration of HMECs through extracellular matrix (Matrigel) was increased upon the treatment of rHsp90α and decreased with the treatment of Hsp90α mAb (Fig. 5B and supplemental Fig. S5B). Moreover, with the knockdown of MMP-2 in HMECs (supplemental Fig. S5A), the pro-transmigration effect of rHsp90α was almost completely abrogated (Fig. 5B and supplemental Fig. S5B). Similar results were also obtained in the tube formation assay in vitro (Fig. 5C).

To further confirm the direct effect of the interaction between Hsp90α and MMP-2 in promoting angiogenesis, a Matrigel plug assay with lentivirus delivered MMP-2 shRNA was employed to examine the role of Hsp90α in angiogenesis in vivo. The knockdown and infectious efficiency of the lentivirus delivered MMP-2 shRNA was examined and shown in
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Hsp90α stabilizes MMP-2 and promotes the transmigration and tube formation of endothelial cells in vitro and in vivo. A, rHsp90α, rHsp90β, mouse IgG, Hsp90α mAb, or Hsp90β mAb (5 μg/ml) was added to the culture medium (without FCS) of HMECs. After 24 h of treatment, the CM was collected and concentrated by 10-fold. MMP-2 and its fragments were detected by anti-MMP-2 antibody. B, the transmigration of HMECs (transfected with MMP-2 shRNA or not) treated by Hsp90 recombinant proteins or antibodies (5 μg/ml) was examined by Matrigel transmigration assay in Millicell chambers. The transmigrated cells were stained by hematoxylin and eosin and counted. The error bars represent S.D. (n = 5), p value: Student’s t test. *, p < 0.05; **, p < 0.01; #, p > 0.05. C, the tube formation of HMECs (transfected with MMP-2 shRNA or not) treated by Hsp90 recombinant proteins or antibodies (5 μg/ml) were examined by Matrigel model in vitro. The error bars represent S.D. (n = 5), p value: Student’s t test. *, p < 0.05; **, p < 0.01; #, p > 0.05. D, the effects of Hsp90 recombinant proteins or antibodies (50 μg/ml) on tube formation in Matrigel plug containing lentivirus delivered MMP-2 shRNA or not were examined in nude mice. The tube formation was detected by immunofluorescence using CD31 staining. The density of the tube was calculated by NIS-Elements C, the software of Nikon A1 confocal. The error bars represent S.D. (n = 5), p value: Student’s t test. *, p < 0.05; **, p < 0.01; #, p > 0.05. MW, molecular mass.

supplemental Fig. S6, A and B. The tube formation in Matrigel plug was detected by immunofluorescence staining with anti-CD31 antibody. The result showed that Hsp90α but not Hsp90β promoted angiogenesis in an MMP-2-dependent manner (Fig. 5D and supplemental Fig. S6C), whereas the antibody of Hsp90α suppressed angiogenesis remarkably (Fig. 5D and supplemental Fig. S6C). Taken together, these results demonstrate that Hsp90α can promote angiogenesis via stabilizing MMP-2.

Hsp90α Promotes Tumor Angiogenesis and Growth in Vivo—Because we found that Hsp90α can be secreted by endothelial cells and promotes endothelial cell transmigration and tube formation in vitro and in vivo, we proposed that Hsp90α may also be involved in the angiogenesis of tumors in vivo. We next examined the effect of Hsp90α mAb on tumor growth and angiogenesis using the B16/F10 melanoma mouse model. Hsp90β mAb was taken as the control protein. The results indicate that Hsp90α mAb but not Hsp90β mAb can suppress tumor growth in a dose-dependent manner (Fig. 6A and B). Furthermore, the blood vessel density of the different groups was assessed using CD31 staining, which showed a significant decrease upon the treatment of Hsp90α mAb but not Hsp90β mAb (Fig. 6C). Collectively, the above results suggest that the antibody of Hsp90α is a potential therapeutic agent for the inhibition of not only tumor metastasis (8–10) but also tumor angiogenesis and growth.

Because Hsp90α mAb remarkably inhibits tumor angiogenesis, we wondered whether the direct localization of Hsp90α mAb on tumor blood vessels can be observed in vivo. Therefore the localization of the administrated antibodies in tumor tissues was detected using the FITC-conjugated anti-mouse IgG antibody. In the groups treated with control mouse IgG or Hsp90β mAb, no specific staining was observed in the tumor tissues, whereas Hsp90α mAb was specifically localized on the tumor blood vessels and the periphery of the tumor cells (Fig. 6D), reflecting the localization of endogenous Hsp90α on the same sites and its important role in modulating tumor angiogenesis.

Next we compared the level of active MMP-2 in tumors treated with Hsp90 monoclonal antibodies by Western
Consistently, the antibody of Hsp90α/H9251 but not Hsp90α/H9252 promoted the degradation of MMP-2. The total amounts of pro-activated and activated MMP-2 were decreased in the tumors treated with Hsp90α/H9251 mAb but not Hsp90α/H9252 mAb (Fig. 6E). These results further confirm that Hsp90α can actually stabilize and enhance the activity of MMP-2 in vivo, which subsequently promotes tumor angiogenesis and invasiveness.

FIGURE 6. The effect of Hsp90α antibody on tumor growth and angiogenesis in the B16/F10 melanoma mouse model. Mouse IgG, Hsp90α mAb (5 or 10 mg/kg), or Hsp90β mAb (5, 10 mg/kg) was injected intraperitoneal for 25 days. A, the tumor growth was monitored every 5 days from the fifth day after the tumor implantation. B, the tumor mass was measured after the sacrifice. C, the blood vessel density in tumor tissue was assessed by immunofluorescence using anti-CD31 antibody. Representative images are shown on the left. Scale bar, 100 μm. The quantified result was analyzed by NIS-Elements C, software of Nikon A1 confocal. The error bars represent S.D. (n = 5). p value: Student’s t test. *, p < 0.05; **, p < 0.01. D, the localization of administered Hsp90α mAb or Hsp90β mAb in tumor tissues was detected by FITC-conjugated anti-mouse IgG. Anti-CD31 antibody was used for detecting blood vessels. Scale bar, 20 μm. E, the processing of MMP-2 in tumor tissue lysate from the mice treated with Hsp90α antibodies was detected by Western blotting. MW, molecular mass.
DISCUSSION

The Regulatory Mechanism of Hsp90α on MMP-2 Processing—The activity of MMP-2 is regulated at four levels: gene expression, compartmentalization (localization), proenzyme (zymogen) activation, and enzyme inactivation (inhibition or processing) (25). The mechanism of ProMMP-2 activation (26) and the inhibition of activated MMP-2 (27) are relatively clear, but the regulation of MMP-2 processing is still poorly understood.

In this study, we demonstrate that the processing of MMP-2 is regulated by extracellular Hsp90α (Figs. 1 and 2). Many substrates or clients of Hsp90α are structurally labile signaling proteins (3). In the absence of Hsp90α binding, these proteins are susceptible to aggregation or degradation (1, 3, 4). Structural variability was also found in MMPs. Almost all of the MMPs possess two terminal globular domains (the catalytic domain and the hemopexin domain) connected by an unstructured linker. Recent studies have found that the linker is flexible and facilitates the conformational change of MMPs from a compact structure into an elongated one (28–30). Although not experimentally confirmed, a similar conformational freedom was also observed by molecular dynamics simulation in MMP-2 (31). Upon the conformational change of MMP-2 from a compact structure to an elongated one (28–30). Although not experimentally confirmed, a similar conformational freedom was also observed by molecular dynamics simulation in MMP-2 (31). Upon the conformational change of MMP-2 from a compact structure to an elongated one, its linker domain becomes loose and exposed, which may facilitate the access and cleavage by degrading enzymes. In our study, using N-terminal sequencing, we verified a cleavage site of MMP-2 processing protected by Hsp90α, which is Glu443–Leu444 and locates in the linker domain of MMP-2 (supplemental Fig. S7A). Interestingly, a similar cleavage mechanism was also observed in the hinge domain of MT1-MMP, which contains a highly exposed loop and is the prime target for proteolysis (32). The aforementioned result provides evidence that this conformational change induces the instability of MMP-2. In the presence of Hsp90α, which is demonstrated here to bind with MMP-2 via the hemopexin domain, the elongated structure would be stabilized, and the cleavage site would be shielded. A hypothetical model showing how Hsp90α mediated the stabilization of MMP-2 is shown in supplemental Fig. S7B.

The Interaction of Hsp90α and MMP-2—Previous studies have shown that Hsp90α interacts with MMP-2 and that the impermeable inhibitor of Hsp90α (immobilized geldanamycin) decreases the activity of MMP-2 (8). In this study, we demonstrate that Hsp90α interacts with the C-terminal hemopexin domain of MMP-2, and this binding protects MMP-2 from inactivation processing (Figs. 1 and 2). Furthermore, Hsp90α antibody promotes the inactivation processing of MMP-2 (Figs. 1D, 5A, and 6E), which is consistent with the effect of the impermeable Hsp90 inhibitor reported previously (8). Therefore, our findings provide a novel mechanistic explanation for the regulation of MMP-2 activity by Hsp90α, which is attributed to the stabilization effect of Hsp90α on the processing of MMP-2.

In addition, we observed that the stabilization effect of Hsp90α on MMP-2 is ATP-independent (supplemental Fig. S3C). Hsp90α contains an ATPase domain, and the hydrolysis of ATP is essential for Hsp90α to bind with its cochaperones and various client proteins in the cytosol (18). However, it has also been observed that ATP is not required for Hsp90α to exert its holding and stabilization functions on partially unfolded proteins (33, 34); our results are consistent with this aspect. It is proposed that ATP binding and hydrolysis may only be essential for Hsp90α chaperoning machinery that requires the interplay of Hsp90α with its cochaperones, regulators, and clients (2). On the other hand, extracellular Hsp90α was identified to be hyperacetylated, which attenuates its binding to ATP but not to MMP-2 (35). Very recently, our group reported that extracellular Hsp90α is phosphorylated at Thr90, which is located in the ATP-binding pocket and may also influence the binding of ATP to Hsp90α (9). These reports all indicate that the function of extracellular Hsp90α is ATP-independent.

Besides, although ATP is not essential for the activity of extracellular Hsp90α, geldanamycin and its derivatives, the inhibitors of Hsp90α that act by blocking ATP binding (17, 19), are still able to bind with extracellular Hsp90α (35). Then their inhibitory effects on MMP-2 activity and tumor invasiveness (8) may be attributed to the attenuation of client binding.

Extracellular Hsp90α Is a Substrate of MMP-2—As an exosite, the hemopexin domain determines the interaction of several substrates to MMP-2 (37). Extracellular Hsp90α, which is identified to be a hemopexin domain-binding protein here (Fig. 3, A and B), is a highly probable substrate of MMP-2. In fact, the cleavage of Hsp90α by MMP-2 was detected previously by analyzing the substrate degradation of MMP-2 (38). In our work, the degradation of Hsp90α upon the treatment of MMP-2 was also observed (supplemental Fig. S4A), and the major degradation products (~50 kDa) were consistent with the previous report (38). More interestingly, Hsp90β, which is quite similar to Hsp90α (6, 39), is actually extremely unstable upon MMP-2 treatment. It can be degraded completely within 1 h at 37 °C by equal molar amounts of MMP-2 (supplemental Fig. S4B), whereas Hsp90α is degraded much more slowly under the same condition (supplemental Fig. S4A). These results indicate that the proteolysis mechanisms of MMP-2 on Hsp90α and Hsp90β are quite different, which may be determined by both the amino acid sequences and the secondary structures of these two isoforms. Although we have detected the cleavage of Hsp90β by MMP-2 by an in vitro noncell system, whether it is a natural substrate of MMP-2 remains to be determined. Ironically, an unfair game appears to exist here: on one hand, Hsp90α stabilizes MMP-2 and prevents it from inactivation processing, but MMP-2 degrades Hsp90α. This observation once again illustrates that the biological system is nothing if not complex.

Extracellular Hsp90α, Angiogenesis, and Tumor Microenvironment—Because intracellular Hsp90α is essential for the stability of diverse cell signaling pathways of cancer cells (40), the inhibitors of Hsp90 can suppress tumor progression by targeting almost all hallmarks of cancer cells, including angiogenesis (41–43). On the other side, extracellular Hsp90α was considered to play a unique role in tumor metastasis (7). In our study, extracellular Hsp90α is demonstrated
for the first time to be a positive regulator of tumor angiogenesis (Figs. 5 and 6), signifying the potential role of Hsp90α antibody on the inhibition of tumor angiogenesis and growth.

In addition, along with the discovery of additional extracellular Hsp90α client proteins (44–46), more functions of extracellular Hsp90α will be revealed. We propose that Hsp90α is not only a biochemical buffer for the genetic lesions of tumor cells in the cytosol but is also essential for the homeostasis of entire tumor microenvironment. Consequently, the applications of Hsp90α antibody or impermeable inhibitors of Hsp90α will be extended and not only limited to the inhibition of metastasis. Moreover, it was shown that other molecular chaperones, such as Hsp70, GRP78, and GRP94/gp96, also exist extracellularly and are involved in the regulation of angiogenesis (36, 47, 48) or other components of tumor microenvironment (7). The applications of these extracellular chaperones as therapeutic targets in cancer treatment all merit further investigation.

In summary, our results demonstrate for the first time that extracellular Hsp90α stabilizes MMP-2 via the interaction of the middle domain of Hsp90α and the hemopexin domain of MMP-2, providing novel mechanistic explanations for both the function of extracellular Hsp90α and the regulatory mechanism of MMP-2 activity. Furthermore, we reveal that Hsp90α can be secreted by endothelial cells and promotes angiogenesis in vitro and in vivo, whereas the antibody of Hsp90α is a potential anti-tumor drug targeting not only metastasis but also angiogenesis.

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