Disturbance of Ca\(^{2+}\) Homeostasis Converts Pro-Met into Non-canonical Tyrosine Kinase p190Met\(^{\text{NC}}\) in Response to Endoplasmic Reticulum Stress in MHCC97 Cells\(^{\text{S1}}\)

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**Background:** Both ER stress and c-Met are implicated in the tumorigenesis of HCC.

**Results:** ER calcium disturbance-induced p190Met\(^{\text{NC}}\) expression inhibits ER stress-mediated apoptosis.

**Conclusion:** p190Met\(^{\text{NC}}\), but not p190Met\(^{\text{αβ}}\), plays a critical role in promoting HCC cells survival under ER stress.

**Significance:** We identify a novel biochemical mechanism by which c-Met ensures HCC cell survival under stress conditions.

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C-Met, the tyrosine-kinase receptor for hepatocyte growth factor, plays a critical role in the tumorigenesis of hepatocellular carcinoma (HCC). However, the underlying mechanism remains incompletely understood. The mature c-Met protein p190Met\(^{\text{αβ}}\) (consists of a α subunit and a β subunit) is processed from pro-Met. Here we show that pro-Met is processed into p190Met\(^{\text{NC}}\) by sarco/endoplasmic reticulum calcium-ATPase (SERCA) inhibitor thapsigargin. p190Met\(^{\text{NC}}\) compensates for the degradation of p190Met\(^{\text{αβ}}\) and protects human HCC cells from apoptosis mediated by endoplasmic reticulum (ER) stress. In comparison with p190Met\(^{\text{αβ}}\), p190Met\(^{\text{NC}}\) is not cleaved and is expressed as a single-chain polypeptide. Thapsigargin-initiated p190Met\(^{\text{NC}}\) expression depends on the disturbance of ER calcium homeostasis. Once induced, p190Met\(^{\text{NC}}\) is activated independent of hepatocyte growth factor engagement. p190Met\(^{\text{NC}}\) contributes to sustained high basal activation of c-Met downstream pathways during ER calcium disturbance-mediated ER stress. Both p38 MAPK-promoted glucose-regulated protein 78 (GRP78) expression and sustained high basal activation of PI3K/Akt and MEK/ERK are involved in the cytoprotective function of p190Met\(^{\text{NC}}\). Importantly, the expression of p190Met\(^{\text{NC}}\) is detected in some HCC cases. Taken together, these data provide a potential mechanism to explain how c-Met promotes HCC cells survival in response to ER stress. We propose that context-specific processing of c-Met protein is implicated in HCC progression in stressful microenvironments.

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The endoplasmic reticulum (ER)\(^{\text{S5}}\) is the primary site for secreted and membrane-bound proteins processing. Disulfide bond formation and glycosylation are two critical steps in the maturation of the majority of the proteins that traffic through the ER (1). The homeostasis of ER environment is essential for the cell to maintain protein homeostasis. Perturbation of ER homeostasis by disruption of calcium (Ca\(^{2+}\)) homeostasis and redox status leads to stress and activation of the unfolded protein response (UPR) (2–4). The UPR is a pro-survival response to reduce the accumulation of misfolded proteins and restore ER function (5–7). If there is a failure to resolve ER stress, the UPR signaling switches from prosurvival to proapoptotic (8–11).

Three major UPR transmembrane sensors, including transcription factor activating transcription factor 6 (ATF6), protein kinase RNA-like ER kinase (PERK), and inositol-requiring enzyme 1 (IRE1), are involved in the UPR signaling. In non-stressed mammalian cells, glucose-regulated protein 78 (GRP78, a highly abundant ER chaperone) constitutively binds to ATF6, PERK, and IRE1 and maintains them in an inactive status. Upon ER stress, sequestration of GRP78 by unfolded proteins activates these sensors and initiates the UPR (12, 13). It is well known that GRP78 plays a critical cytoprotective role against ER stress (13–17).

C-Met, the receptor for hepatocyte growth factor (HGF), is a transmembrane tyrosine kinase. C-Met is initially synthesized as a partially glycosylated single-chain precursor (pro-Met, 170-kDa). pro-Met undergoes disulfide bonds formation, post-translational glycosylation, and endoproteolytic cleavage to produce the mature heterodimeric form of c-Met (p190Met\(^{\text{αβ}}\)) (18). p190Met\(^{\text{αβ}}\) consists of a 50-kDa α subunit (p50Met) and a

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\(^{\text{S2}}\)The abbreviations used are: ER, endoplasmic reticulum; SERCA, sarco/endoplasmic reticulum calcium-ATPase; GRP78, glucose-regulated protein 78; UPR, unfolded protein response; ATF6, activating transcription factor 6; PERK, protein kinase RNA-like ER kinase; HGF, hepatocyte growth factor; HCC, human hepatocellular carcinoma; PBA, 4-phenyl butyric acid; XBP1, X-box-binding protein 1; BHQ, 1,4-dihydroxy-2,5-di-tert-butylbenzene; Tg, thapsigargin; Tun, tunicamycin; CHX, cycloheximide.

\(^{\text{S3}}\)This article contains supplemental Figs. S1–S8 and Table S1.

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145-kDa β subunit (p145Met) that are joined by disulfide bonds. The α subunit is entirely extracellular, whereas the β subunit spans the membrane and contains the tyrosine kinase catalytic domain (18). The phosphorylation on Tyr-1234/1235 residues in the catalytic domain is crucial for c-Met activation, whereas phosphorylation on Tyr-1349/1356 residues are essential for c-Met to form the docking site and activate the downstream signaling cascades (19). Deregulation of HGF/c-Met signaling is implicated in the development and metastasis of many types of human tumor, such as human hepatocellular carcinoma (HCC) (20–25). It has been accepted that aberrant activation of c-Met is strongly associated with overexpression of the receptor without HGF engagement (23, 26).

Due to poor vascularization and rapid growth, solid tumors are constantly or intermittently exposed to stressful microenvironments, including hypoxia and nutrient starvation. Both hypoxia and nutrient starvation are strong inducers for ER stress. (27). Appropriate ER stress response plays an important role in protecting cancer cells against harmful microenvironments. Previous studies have demonstrated that ER stress is involved in the progression of HCC (28–30).

In this study we investigated the potential association between c-Met and ER stress in HCC. We found that ER calcium disturbance converts pro-Met into a more stable functional form of c-Met (p190Met NC). p190Met NC inhibits prolonged ER stress-induced apoptosis through sustaining high basal activities of PI3K/Akt and MEK/ERK survival pathways. Furthermore, phosphorylated p190Met NC is detected in HCC cases. These data indicate that pro-Met is processed into a distinct functional form of c-Met to ensure HCC cell survival under ER stress circumstances.

MATERIALS AND METHODS

Human Tissues—Human liver biopsies were obtained from Shanghai Eastern Hepatobiliary Surgery Hospital. This study has been approved by the local ethical committee.

Chemicals and Antibodies—ER stress inducers dithiothreitol (DTT), disruptor of disulfide bonds, thapsigargin (inhibitor of sarco/endoplasmic reticulum Ca$^{2+}$-ATPase, SERCA), and tunicamycin (inhibitor of N-linked glycosylation), ER stress inhibitor PBA, N-linked glycosylation inhibitor peptide N-glycosidase F, and protein synthesis inhibitor CHX were purchased from Sigma. The c-Met inhibitor PF-2341066 was purchased from Selleck Chemicals. The SERCA inhibitor 1,4-dihydroxy-2,5-dimethyl-6-butylenzene (BHQ) and c-Met inhibitor SU11274 were purchased from Tocris Bioscience. The P3k inhibitor LY294002, MEK inhibitor U0126, and p38 MAPK inhibitor SB203580 were purchased from Merck. The elF2α dephosphorylation inhibitor salubrin, c-Met siRNA, ATF4 siRNA, and antibodies against c-Met, GRP78, ATF6, elF2α, and β-actin were purchased from Santa Cruz Biotechnology. Antibodies against phospho-Akt (Ser-473), phospho-ERK (Thr-202/Tyr-204), phospho-Met (Tyr-1234/1235, Tyr-1349), phospho-p38 MAPK (Thr-180/Tyr-182), phospho-elF2α (Ser-51), Akt, ERK, p38 MAPK, ATF4, poly(ADP-ribose) polymerase, and Cleaved Caspase-3 were purchased from Cell Signaling Technology. Antibody against IR was purchased from Bioworld Technology Corp.

Cell Culture and Treatments—Human HCC cell lines MHCC-97H, MHCC-97L, SMMC-7721, HepG2, PLC, and Huh-7 were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified incubator containing 5% CO$_2$ and 95% ambient air at 37 °C. The protocol used for c-Met and ATF4 knockout has been previously described (28).

Immunoprecipitation and Western Blot—Cells were harvested in the appropriate buffer for the purpose of each experiment. Protein concentrations were measured using the BCA assay (Santa Cruz Biotechnology). Protein samples were denatured with 4× SDS-loading buffer (200 mM Tris, pH 6.8, 8% SDS, 400 mM DTT, 0.4% bromphenol blue, 40% glycerol) at 100 °C for 5 min and subjected to standard SDS-PAGE and Western blot analysis as previously described (28).

RT-PCR Analysis—Total RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The reverse transcription reactions were carried out using the M-MLV reverse transcriptase (Promega) according to the manufacturer’s protocol. The gel images were digitally captured with the SynGene gel documentation system and analyzed with the Genetools analysis software (Syngene, Frederick, MD). All tests were repeated three times, and one of the repeats was shown in the results. The primers used in this study are shown in supplemental Table 1.

Apoptosis Analysis—Apoptosis was detected using the annexin V-FITC apoptosis detection kit (Invitrogen) according to the manufacturer’s manual. Annexin V staining was analyzed by flow cytometry within 1 h. The experiments were repeated three times.

Deglycosylation—Deglycosylation was performed according to the manufacturer’s manual. A denaturation process was required for the deglycosylation of glucose oxidase. For treatment with peptide N-glycosidase F, 20 μl of G7 buffer, 20 μl of 10% Nonidet P-40, and 5 μl of peptide N-glycosidase F were added, and the mixture was incubated at 37 °C for 12 h. The deglycosylated protein samples were subjected to standard SDS-PAGE and Western blot analysis.

Proteolytic Digestion—Mild trypsin digestion was performed as previously described (31). Cells were washed with phosphate-buffered saline and incubated in serum-free Dulbecco’s modified Eagle’s medium with 100 μg/μl trypsin. Digestion was carried out at 37 °C in a humidified incubator containing 5% CO$_2$ and 95% ambient air for 20 min.

Statistical Analysis—Results were expressed as the mean ± S.D. Statistical analysis was performed using Student’s t test. p < 0.05 was considered statistically significant.

RESULTS

Effect of ER Homeostasis Disturbance on c-Met Expression—To investigate the effect of ER homeostasis disturbance on c-Met expression, we first examined the protein levels of c-Met in MHCC97-H, MHCC97-L, SMMC-7721, PLC, Huh-7, and HepG2 human HCC cell lines. As shown in Fig. 1A, MHCC97-H and MHCC97-L cells showed strong expression and constitutive phosphorylation of c-Met. Incubation of SMMC-7721, MHCC97-H, and MHCC97-L cells with ER stress inducers dithiothreitol, thapsigargin, and tunicamycin

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accumulation (supplemental Fig. 1, effect on dithiothreitol- and tunicamycin-induced pre-Met administration (Fig. 1, additional 190-kDa band was observed after thapsigargin treatment resulted in pro-Met and nonglycosylation are essential for pro-Met processing and maturation. Thapsigargin-induced 190-kDa molecular might be another molecular is encoded by c-Met proto-oncogene. c-Met knockdown (Fig. 2, A) indicated the spliced form (active form) and unspliced form (inactive form) of XBP1 mRNA, respectively. C and D, shown is the effect of ER stress on c-Met expression. MHCC97-H and MHCC97-L cells were treated with thapsigargin (1 μM), dithiothreitol (2.5 mM), and tunicamycin (2.5 μg/ml) for the indicated time periods and then subjected to RT-PCR analysis. The shorter band (S) and longer band (L) indicated the spliced form (active form) and unspliced form (inactive form) of XBP1 mRNA, respectively. C and D, shown is the effect of ER stress on c-Met expression. MHCC97-H and MHCC97-L cells were treated with thapsigargin (1 μM), dithiothreitol (2.5 mM), and tunicamycin (2.5 μg/ml) for the indicated time periods and then subjected to Western blot analysis.

FIGURE 1. The effect of ER homeostasis disturbance on c-Met expression. A, shown is Western blot analysis of phosphorylation and protein levels of c-Met in human HCC cell lines. PLC, protein lipase C. B, dithiothreitol, thapsigargin, and tunicamycin induce the UPR. SMMC-7721, MHCC97-H, and MHCC97-L cells were treated with dithiothreitol (2.5 mM), thapsigargin (1 μM), and tunicamycin (2.5 μg/ml) for indicated time periods and then subjected to RT-PCR analysis. The shorter band (S) and longer band (L) indicated the spliced form (active form) and unspliced form (inactive form) of XBP1 mRNA, respectively. C and D, shown is the effect of ER stress on c-Met expression. MHCC97-H and MHCC97-L cells were treated with thapsigargin (1 μM), dithiothreitol (2.5 mM), and tunicamycin (2.5 μg/ml) for the indicated time periods and then subjected to Western blot analysis.

rapidly induced the expression or activation of the UPR biomarkers (Fig. 1B). The biomarkers of UPR activation included GRP78 and growth arrest and DNA damage-inducible protein 153 (GADD153) mRNA induction and X-box-binding protein 1 (XBP1) mRNA splicing (Fig. 1A). As the proteins were separated by SDS-PAGE under reducing conditions, it is reasonable to indicate that the 190-kDa c-Met detected by Western blot is a single-chain polypeptide. To make sure is the 190-kDa c-Met is the single-chain polypeptide p190MetNC reported by Mondino et al. (31), thapsigargin-treated MHCC97-H and MHCC97-L cells were incubated with trypsin under mild conditions (31). After trypsin digestion, the 190-kDa c-Met initiated by thapsigargin was cleaved into α and β subunits (Fig. 2F), indicating that the 190-kDa c-Met is the single-chain polypeptide p190MetNC.

As disturbance of ER Ca2+ homeostasis induces ER stress, we addressed whether the UPR participates in thapsigargin-mediated p190MetNC induction. ER stress inhibitor PBA (32) preincubation had no apparent effect on thapsigargin-induced p190MetNC expression (Fig. 2G), indicating that the UPR is not involved in ER Ca2+ depletion-initiated p190MetNC expression. The proteasome inhibitor MG132 induced ER stress without p190MetNC induction (supplemental Fig. 2E), confirming that p190MetNC is not induced by the UPR. Thus, ER Ca2+ homeostasis disturbance might attribute to p190MetNC induction. The role of disturbance of ER Ca2+ homeostasis in p190MetNC induction was further supported by the data that BHQ, another selective inhibitor of SERCA, stimulated the expression of p190MetNC (supplemental Fig. 2A). Moreover, BHQ-initiated p190MetNC was cleaved into α and β subunits by trypsin digestion, and PBA preincubation had no apparent effect on p190MetNC expression upon BHQ treatment (supplemental Fig. 2B, B and C). Taken together, these results show that disturbance of ER Ca2+ homeostasis initiates pro-Met processed into p190MetNC.
**p190MetNC Responsible for Constitutive High Basal Activation of Downstream Pathways under ER Stress**—As PI3K/Akt and MEK/ERK are two typical downstream pathways of c-Met (22, 23), we investigated whether the phosphorylation of Akt and ERK are c-Met-dependent in c-Met positive HCC cells. The results showed that the phosphorylation of Akt and ERK were c-Met-dependent in MHCC97-H and MHCC97-L cells under physiological and ER stress conditions (Fig. 3, A and B). It has been reported that long term exposure to ER stress stimuli results in gradually increasing ERK activity but decreasing Akt activity (33–36). The phosphorylation kinetics of Akt and ERK in thapsigargin- and dithiothreitol-treated MHCC97-H and MHCC97-L cells were investigated. In comparison with SMMC-7721, high basal levels of Akt and ERK phosphorylation were sustained for at least 24 h in MHCC97-H and MHCC97-L cells in response to thapsigargin (Fig. 3C). However, dithiothreitol treatment led to a rapid reduction in the basal phosphorylation levels of Akt and ERK in MHCC97-H and MHCC97-L cells (Fig. 3D). In MHCC97-H and MHCC97-L cells, the phosphorylation levels of Akt and ERK significantly decreased after dithiothreitol incubation for 48 h in comparison with thapsigargin treatment (Fig. 3E). As the phosphorylation of Akt and ERK are c-Met-dependent in response to thapsigargin (Fig. 3A) and dithiothreitol (Fig. 3B), it is possible that thapsigargin and dithiothreitol exert different effects on the phosphorylation of Akt and ERK through c-Met regulation. This notion is supported by the data that PF-2341066 not only blocked the phosphorylation of p190MetNC but also decreased the phosphorylation levels of Akt and ERK after thapsigargin treatment (Fig. 3F). The role of p190MetNC in sustaining high basal phosphorylation levels of Akt and ERK under ER stress conditions was confirmed by the suppression of c-Met expression (Fig. 3G). Taken together, these results suggest that p190MetNC is responsible for sustained high basal activation levels of c-Met downstream pathways under ER stress conditions.

**p190MetNC Promotes HCC Cell Survival under ER Stress**—To determine whether c-Met is implicated in protecting HCC cells against ER Ca\(^{2+}\) homeostasis disturbance-initiated ER stress, PF-2341066 was used to blocked c-Met activity. Compared with SMMC-7721 (supplemental Fig. 3A), PF-2341066 preincubation promoted thapsigargin-induced apoptosis in MHCC97-H and MHCC97-L cells (Fig. 4A). This cytoprotective effect of c-Met was confirmed by c-Met knockdown (Fig. 4B). Similar results were found when MHCC97-H and MHCC97-L cells were treated by BHQ with or without PF-2341066 preincubation (data not shown). These data reveal a cytoprotective function for c-Met in response to ER stress in c-Met-positive HCC cells. As we...
demonstrated that p190MetNC is the major functional form of c-Met after long term thapsigargin treatment, it is reasonable to suggest that the major cytoprotective function for c-Met during persistent ER stress is dependent on p190MetNC but not p190MetH9251/H9252.

Considering the cytoprotective role of UPR activation under ER stress condition (3), we investigated the effect of c-Met activity blocking on the three major UPR specific signaling pathways, including PERK/eIF2α, ATF6, and XBP1. As shown in supplemental Fig. 3B, PF-2341066 preincubation had no demonstrable effect on the activation of PERK/eIF2α and ATF6 pathways. Furthermore, blocking the activity of c-Met had no significant effect on the splicing of XBP-1 mRNA (supplemental Fig. 3C). These results suggest that the UPR-specific pathways mediated by PERK/eIF2α, ATF6, and XBP1 are not involved in the cytoprotective role of p190MetNC upon ER stress.

As PI3K/Akt and MEK/ERK have been shown to prevent ER stress-induced cell death (28, 33) and the activity of Akt and ERK in thapsigargin- and dithiothreitol were shown. SMMC-7721, MHCC97-H, and MHCC97-L cells were treated with thapsigargin (1 μM) and dithiothreitol (2.5 mM) for the indicated time periods and then subjected to Western blot analysis. As our data demonstrate that PF-2341066 treatment inhibited both PI3K/Akt and MEK/ERK phosphorylation, we introduced LY294002 and U0126 to selectively inhibit PI3K and MEK, respectively. The data showed that neither PI3K nor MEK inhibition had an apparent effect on thapsigargin- and salubrinal-mediated GRP78 induction (supplemental Fig. 4A and B).
As the p38 MAPK pathway, one of c-Met downstream pathways (37), is involved in the induction of GRP78 under ER stress conditions (38), we addressed whether p38 MAPK participates in c-Met inhibition-mediated GRP78 down-regulation. As depicted in Fig. 5C, p38 MAPK inhibitor SB203580 pretreatment inhibited GRP78 induction. Furthermore, blocking c-Met activity inhibited the phosphorylation of p38 MAPK upon thapsigargin treatment (Fig. 5D). These data suggest that the inactivation of p38 MAPK contributes to c-Met blocking-mediated GRP78 down-regulation.

To make sure whether p190MetNC is critical for thapsigargin-mediated GRP78 induction, dithiothreitol was used in our study. In comparison with thapsigargin, dithiothreitol treatment decreased the phosphorylation of p38 MAPK (Fig. 5E). More importantly, dithiothreitol-mediated GRP78 induction was much lower than that of thapsigargin (Fig. 5E). However, thapsigargin and dithiothreitol had no apparent difference in GRP78 induction in c-Met-negative HCC cell lines SMMC-7721 and PLC (supplemental Fig. 5). Furthermore, blocking p190MetNC activation with PF-2341066 not only decreased the phosphorylation levels of p38 MAPK but also inhibited GRP78 induction in MHCC97-H and MHCC97-L cells after thapsigargin treatment (Fig. 5E). The role of p190MetNC in promoting GRP78 induction was confirmed by the suppression of c-Met expression (Fig. 5F). Together, these data suggest that p190MetNC is essential for GRP78 induction in response to thapsigargin.

p190MetNC Compensates for p190MetΔH Down-regulation—Because ER Ca2+ homeostasis disturbance decreased both phosphorylation and protein levels of p145Met (β subunit
of p190MetNC with a concomitant induction of p190MetNC, it is interesting to investigate the difference of the stability of the two forms of c-Met upon ER stress. Importantly, p190MetNC was more stable than p145Met upon thapsigargin treatment (Fig. 6A). As shown in supplemental Fig. 6A, eIF2α inhibitor salubrinal had no demonstrable effect on the protein level of p145Met. Moreover, the mRNA level of c-Met did not change during thapsigargin treatment (supplemental Fig. 6B). Thus, thapsigargin promoted p145Met down-regulation through regulating its stability. Because the binding of HGF to c-Met initiates the degradation of c-Met (39), we propose that the constitutive activation of c-Met may be a driver of p145Met decreasing under ER stress. This speculation was demonstrated by the findings that PF-2341066 pretreatment significantly inhibited thapsigargin-triggered p145Met degradation (Fig. 6D). These data indicate that the phosphorylation on Tyr-1234/1235/1349 is crucial for ER stress-mediated p145Met degradation. These results, when taken together, indicate that p190MetNC compensates, at least in part, for p190MetNC in response to the disturbance of ER Ca²⁺ homeostasis.

Expression of p190MetNC in HCC—To investigate whether the association between ER stress and p190MetNC is involved in the progression of HCC, we examined the expression of c-Met in 27 HCC cases. As shown in Fig. 7A, the expression of p190MetNC and GRP78 proteins was detected in 6 and 18 of 27 HCC cases, respectively. The expression of p190MetNC was correlated with high protein levels of ER stress marker GRP78 (Fig. 7A). Furthermore, p190MetNC was phosphorylated in HCC tissues (Fig. 7B). Immunoblotting of c-Met immunoprecipitates from HCC tissues and thapsigargin-treated MHCC97-L cells with antibody against c-Met confirmed the expression of p190MetNC in HCC cases (Fig. 7C). Moreover, the up-regulation of c-Met mRNA was detected in some cases of HCC, including p190MetNC-expressed cases (Fig. 7D). Thus, p190MetNC might be implicated in the progression of HCC under ER stress conditions. Further studies are needed to investigate the detailed association between p190MetNC and defective ER protein processing in HCC cases.

DISCUSSION

The UPR is an important cytoprotective response for the cell to cope with ER stress, but the UPR will initiate apoptosis in response to prolonged ER stress (8–10). Although the UPR is generally activated in various solid tumors, it is unclear how tumor cells adapt to long term ER stress. We have previously
shown that HCC cells are relatively resistant to ER stress-induced apoptosis (28), but the detailed mechanisms remain largely unknown. The present work reveals that a non-canonical form of c-Met tyrosine-kinase receptor p190MetNC exerts a potent protective effect against prolonged ER stress mediated by ER Ca\(^{2+}\) disturbance in HCC cells.

Induction of ER stress response resulted in gradually decreasing both the protein and phosphorylation levels of p145Met (c-Met tyrosine kinase subunit). Interestingly, an additional 190-kDa band was detected by antibodies against non-phosphorylated and phosphorylated forms of c-Met in thapsigargin-treated MHCC97-H and MHCC97-L cells. It has been reported that pro-Met from colon carcinoma cell line LoVo is not cleaved and is exposed at the cell surface as a single-chain polypeptide (p190MetNC, 190-kDa) (31). This raises the question of whether thapsigargin-induced 190-kDa band is the single-chain polypeptide p190MetNC. The c-Met siRNA data demonstrated that the 190-kDa band was encoded by c-Met proto-oncogene. As the 190-kDa band was processed from pro-Met and was cleaved into \(\alpha\) and \(\beta\) subunits by trypsin digestion, we suggest that thapsigargin-initiated 190-kDa molecular is p190MetNC.

Because thapsigargin is ER stress inducer, it raises the question of whether the UPR is involved in p190MetNC induction. Based on our data that ER stress inhibitor PBA (32) did not inhibit thapsigargin-mediated p190MetNC induction and MG132 induced UPR without p190MetNC induction, we propose that thapsigargin-mediated p190MetNC expression is UPR-independent. Considering that thapsigargin initiates ER stress through disturbing ER Ca\(^{2+}\) homeostasis, we asked whether disruption of ER Ca\(^{2+}\) homeostasis accounts for the induction of p190MetNC. Based on the data that BHQ, another potent and selective inhibitor of SERCA, treatment stimulated p190MetNC expression independent of the UPR, we suggest that the disturbance of ER Ca\(^{2+}\) stores responsible for thapsigargin-mediated p190MetNC expression. Under physiological conditions, the c-Met mRNA is translated into a precursor (pro-Met) with an apparent molecular mass of 170 kDa. Within a few minutes, pro-Met folds into a structure stabilized by intrachain disulfide bonds. Then, the precursor is glycosylated and

**FIGURE 6.** p190MetNC is more stable than p145Met. A, p190MetNC is more stable than p145Met upon ER stress. To investigate the stability of p145Met, MHCC97-L cells were treated with CHX (10 \(\mu\)M) and thapsigargin (1 \(\mu\)M) for indicated time periods and then subjected to Western blot analysis. To investigate the stability of p190MetNC, MHCC97-L cells were treated with CHX (10 \(\mu\)M) for indicated time periods with thapsigargin (1 \(\mu\)M) preincubation for 24 h and then subjected to Western blot analysis. B, PF-2341066 inhibits thapsigargin-induced p145Met down-regulation. MHCC97-H and MHCC97-L cells were treated with thapsigargin (1 \(\mu\)M) for the indicated time periods with or without PF-2341066 (100 nm) preincubation for 1 h and then subjected to Western blot analysis. C, PF-2341066 increases the stability of p145Met. MHCC97-H and MHCC97-L cells were treated with CHX (10 \(\mu\)M) for the indicated time periods with or without PF-2341066 (100 nm) preincubation for 1 h and then subjected to Western blot analysis. PF-2341066 has no effect on the stability of p190MetNC. To investigate the effect of PF-2341066 on the stability of p190MetNC, MHCC97-H and MHCC97-L cells were treated with CHX (10 \(\mu\)M) for the indicated time periods with or without PF-2341066 (100 nm) preincubation for 1 h and then subjected to Western blot analysis. MHCC97-H and MHCC97-L cells were treated with thapsigargin (1 \(\mu\)M) for 24 h before CHX treatment.
cleaved by a protease to originate p190Met/H9251/H9252 (consists of the H9251 and the H9252 subunits that have been joined by disulfide bonds) (18). As ER is the site for secreted and membrane-bound proteins processing, it is reasonable that disturbance of ER Ca\(^{2+}\) homeostasis inhibits pre-Met cleavage in the ER and initiates the single-chain polypeptide p190MetNC formation. Further studies are needed to investigate the detailed mechanisms that link ER Ca\(^{2+}\) homeostasis disturbance and p190MetNC induction. Both dithiothreitol and tunicamycin blocked ER Ca\(^{2+}\) homeostasis disturbance-mediated p190Met NC induction (supplemental Fig. 2D), indicating that disulfide bond formation and N-linked glycosylation are required for pro-Met processed into p190MetNC. Thus, both p190Met\(^{\alpha\beta}\) and p190MetNC are processed from pro-Met. The difference between p190Met\(^{\alpha\beta}\) and p190MetNC is that p190MetNC is not proteolytically cleaved. However, the difference between pro-Met and p190MetNC is that p190MetNC contains disulfide bonds and posttranslational glycosylation (supplemental Fig. 2D). As the uncleaved-insulin receptor induced by thapsigargin was cleaved into β subunit by trypsin digestion (supplemental Fig. 2, F and G), it is reasonable to suggest that the proteolytic processing generally inhibited by ER Ca\(^{2+}\) homeostasis disturbance.

As p190MetNC is phosphorylated on Tyr-1234/1235 independent of HGF engagement, it is interesting to investigate the function of p190MetNC under ER Ca\(^{2+}\) homeostasis disturbance-induced ER stress. It has been reported that ER stress results in sustained activation of the MEK/ERK pathway but gradual inactivation of the PI3K/Akt pathway (33–36). It is notable that dithiothreitol treatment resulted in PI3K/Akt and MEK/ERK dramatic inactivation in MHCC97-H and MHCC97-L cells. However, thapsigargin administration had no apparent effect on the basal phosphorylation levels of PI3K/Akt and MEK/ERK. As the phosphorylation of PI3K/Akt and MEK/ERK is c-Met-dependent in MHCC97-H and MHCC97-L cells, it is reasonable that the different effects of thapsigargin and dithiothreitol on the PI3K/Akt and MEK/ERK pathways are due to their different roles in c-Met regulating. Both thapsigargin and dithiothreitol decreased p145Met. Compared with dithiothreitol, thapsigargin initiated p190MetNC expression, indicating that p190MetNC might be involved in sustaining high basal phosphorylation levels of PI3K/Akt and MEK/ERK. This speculation is supported by our data which demonstrate that either blocking or knockdown of p190MetNC inhibited the phosphorylation of PI3K/Akt and MEK/ERK in thapsigargin-treated MHCC97-H and MHCC97-L cells. Thus, p190MetNC is responsible for sustained activation of c-Met downstream pathways upon ER stress.

MHCC97-H and MHCC97-L cells express high levels of c-Met compared with SMMC-7721, PLC, Huh-7, and HepG2 cells. It is notable that the resistance of MHCC97-H and MHCC97-L cells to thapsigargin-induced apoptosis was reversed by the blocking of p190MetNC activity with PF-2341066, whereas PF-2341066 had no effect on thapsigargin-induced apoptosis in SMMC-7721 and PLC cells. Similar results were obtained using another selective c-Met inhibi
The knockdown of c-Met also rendered MHCC97-H and MHCC97-L cells more sensitive to thapsigargin-induced apoptosis. We, therefore, conclude that p190MetNC promotes c-Met-positive HCC cells survival under ER stress conditions.

An important question now before us is how p190MetNC promotes HCC cells survival under ER stress conditions. Based on our findings, we propose that c-Met does not directly regulate the major UPR pathways (PERK/eIF2α/Hθ, ATF6, and XBP1 pathways). As ER stress activates non-UPR specific survival pathways such as PI3K/Akt and MEK/ERK in parallel with the UPR pathways, we focused on the role of c-Met in regulating non-UPR specific pathways. Because the PI3K/Akt and MEK/ERK pathways, two downstream signaling pathways of c-Met (22), play a pivotal role in protecting cells against ER stress (28), it is reasonable to suggest that p190MetNC might inhibit thapsigargin-induced apoptosis through the PI3K/Akt and MEK/ERK pathways. p190MetNC inhibition or knockdown led to PI3K/Akt and MEK/ERK inactivation in ER-stressed MHCC97-H and MHCC97-L cells. Furthermore, blocking PI3K substantially increased the sensitivity of MHCC97-H and MHCC97-L cells to thapsigargin. One of the possibilities of c-Met blocking-induced GRP78 down-regulation is through the PI3K/Akt, MEK/ERK, or p38 MAPK pathway. Our results suggest that PI3K or MEK is not involved in c-Met-mediated GRP78 regulation. c-Met inhibition had no demonstrable effect on the induction of spliced XBP1 mRNA, indicating that XBP1 is not involved in c-Met blocking-mediated GRP78 down-regulation. Importantly, blocking the activity of p38 MAPK decreased thapsigargin-mediated GRP78 induction. Moreover, the activity of c-Met is important for sustaining the activity of p38 MAPK under ER stress conditions. Accordingly, p38 MAPK contributes to c-Met-regulated GRP78 induction upon thapsigargin stimulation. Our data further reveal that p190MetNC, but not p190Metαβ, is essential for p38 MAPK-mediated GRP78 induction. One of the mechanisms for the promotive effect of p38 MAPK on GRP78 induction is through promoting the phosphorylation of ATF6, further enhancing its efficacy as an activator to induce GRP78 expression (38). Additionally, c-Met blocking also inhibited salubrinal-mediated GRP78 induction through ATF4 down-regulation (supplemental Fig. 8).

c-Met has been reported to play an important role during the tumorigenesis of HCC (20, 22). Here, we found that the expression of p190MetNC in HCC cases is correlated with ER stress status. Considering that pro-Met was processed into p190MetNC under ER stress conditions, we speculate that only p190MetNC, but not p190Metαβ, was detected in some HCC samples and was due to ER stress status in these HCC samples. Hence, p190MetNC might be a key factor to protect HCC cells from stressful microenvironments, such as ER stress conditions.

**FIGURE 8. Schematic of ER Ca^{2+} homeostasis disturbance-mediated p190MetNC expression and its role in ER stress.** SERCA inhibitor-induced ER Ca^{2+} homeostasis disturbance processed pro-Met into p190MetNC, but not p190Metαβ. In response to ER stress, p190MetNC compensates for p190Metαβ to a certain extent and promotes HCC cells survival.
ER Ca\textsuperscript{2+} Disturbance Initiates p190Met\textsuperscript{NC} Induction

In brief, the present work reveals that pro-Met can be processed into different forms in the context of cell physiology. ER Ca\textsuperscript{2+} homeostasis disturbance initiates pro-Met processing into more stable functional form p190Met\textsuperscript{NC}, which compensates for p190Met\textsuperscript{II} to a certain extent and protects HCC cells against prolonged ER stress (Fig. 8). More detailed studies on the function of c-Met upon ER stress will contribute to the understanding of molecular mechanisms of hepatocarcinogenesis and the development of new therapeutic strategies against HCC.

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