Introduction

Met-hepatocyte growth factor (HGF)/Met system is deregulated in tumors and is implicated in different aspects of invasive growth. Here, we report that in the highly aggressive MDA-MB231 breast carcinoma cells, Met cytosolic fragments (C-terminal fragment [CTF]) were present in the nuclei. They were constitutively active because tyrosine phosphorylated at regulatory and catalytic domains and endowed with transactivating activity independently of HGF exposure. In fact, various constructs containing juxtamembrane (Jxtm) Met fragments, fused with Gal4 DNA-binding domain, transactivated Gal4Luc activity. MDA-MB231 cells were devoid of WW domain-containing oxidoreductase (Wwox) tumor suppressor. Exogenous Wwox protein expression negatively regulated Jxtm3-transactivating activity and decreased spontaneous migration of MDA-MB231 cells. Also, we demonstrate that the lack of endogenous Wwox in MDA-MB231 cells represented a molecular mechanism for intranuclear Met-CTF accumulation and for the decrease of full-length Met stability. Yes-associated proteins maintained constitutively activated nuclear Met fragments that played a role as transcription factors regulating genes probably included those for motile phenotype. The difference with low invasive MCF-7 cells was evident because the latter did not show intranuclear Met and the transactivated constructs-containing Jxtm fragments were inactive also in the presence of HGF. The constitutive activation of nuclear Met-signaling pathway in MDA-MB231 cells, possibly determined at genetic or epigenetic levels of WWOX gene, might participate in breast carcinoma progression influencing invasive/metastatic phenotype. Wwox/Met system can be suggested as a potential target to impair breast carcinoma progression.

Abbreviations: CTF, C-terminal fragment; FBS, fetal bovine serum; GFP, green fluorescent protein; HGF, hepatocyte growth factor; Jxtm, juxtamembrane; RTK, receptor tyrosine kinase; Wwox, WW domain-containing oxidoreductase; YAP, Yes-associated protein.

Nuclear localization of active HGF receptor Met in aggressive MDA-MB231 breast carcinoma cells

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Hepatocyte growth factor (HGF)/Met system is deregulated in tumors and is implicated in different aspects of invasive growth. Here, we report that in the highly aggressive MDA-MB231 breast carcinoma cells, Met cytosolic fragments (C-terminal fragment [CTF]) were present in the nuclei. They were constitutively active because tyrosine phosphorylated at regulatory and catalytic domains and endowed with transactivating activity independently of HGF exposure. In fact, various constructs containing juxtamembrane (Jxtm) Met fragments, fused with Gal4 DNA-binding domain, transactivated Gal4Luc activity. MDA-MB231 cells were devoid of WW domain-containing oxidoreductase (Wwox) tumor suppressor. Exogenous Wwox protein expression negatively regulated Jxtm3-transactivating activity and decreased spontaneous migration of MDA-MB231 cells. Also, we demonstrate that the lack of endogenous Wwox in MDA-MB231 cells represented a molecular mechanism for intranuclear Met-CTF accumulation and for the decrease of full-length Met stability. Yes-associated proteins maintained constitutively activated nuclear Met fragments that played a role as transcription factors regulating genes probably including those for motile phenotype. The difference with low invasive MCF-7 cells was evident because the latter did not show intranuclear Met and the transactivated constructs-containing Jxtm fragments were inactive also in the presence of HGF. The constitutive activation of nuclear Met-signaling pathway in MDA-MB231 cells, possibly determined at genetic or epigenetic levels of WWOX gene, might participate in breast carcinoma progression influencing invasive/metastatic phenotype. Wwox/Met system can be suggested as a potential target to impair breast carcinoma progression.
Wwox for the regulation of transcription factors (17,22), we examined whether YAP1 and 2 might influence the transactivating activity of Met in breast carcinoma cells, expressing or not endogenous Wwox. Here, we report that nuclear Met Jxtm fragments had a transactivating activity in MDA-MB231 cells, independently of HGF treatment. This is the first report showing that Wwox deficiency in aggressive breast carcinoma cells was correlated with nuclear localization of Met-CTF fragments, endowed with transactivating activity and cell invasiveness, while reducing entire Met receptor stability. Endogenous YAPs maintained Met-CTF-constitutive transactivating activity. The pattern of Met in MCF-7 cells was opposite, also because of elevated endogenous Wwox: exogenously expressed YAP1 and 2 increased Jxtm transactivating activity. Intranuclear Met might have a role enhancing signaling of the full-length Met receptor, starting at plasma membrane, or may be indicative of novel signaling pathways in aggressive tumors important for migration in invasive/metastatic phenotype.  

**Materials and methods**

**Materials**

RPMI-1640 and clasto-Lactacyclin β-Lactone (LLNL) were from Sigma Chemical Co. (St Louis, MO). Recombinant human HGF was from R&D Systems (Abingdon, UK). Anti-phospho-Met (Tyr1349) and (Tyr1234/35), anti-phospho-Akt(Ser473) antibodies were from Cell Signaling Technology (Beverly, MA). Anti-phospho-c-Src(Tyr416), conjugated with Alexa Fluor488, and anti-phospho-pyruvate kinase (4G10) antibodies were from Upstate Biotechnology (Lake Placid, NY). Alexa Fluor647 goat anti-rabbit secondary antibody was from Molecular Probes (Eugene, OR). Anti- Met (C12, anti-Wwox (N19), anti-Akt (H136), anti-histone H3, anti-α-tubulin and anti-vinculin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Klenow polymerase and dual luciferase reporter assay system were from Promega (Madison, WI).

**Cell cultures**

Human breast carcinoma cells MCF-7 and MDA-MB231 (European Cell Culture Collection, Salisbury, UK) were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS). Before HGF treatment (200 ng/ml), the cells were starved (0.1% FBS) for 18–24 h (10).

**Confocal laser scanning**

The cells exposed to HGF on coverslips, treated with 1 μM pervanadate for 1 min to evaluate phospho-c-Src signals, fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100, were allowed to react with anti-Met (1:50) and then with Alexa Fluor647 antibodies, followed by exposure to fluorescent anti-phospho-c-Src(Tyr416) antibody (2 μg/ml). For negative control staining, the cells were incubated only with the secondary antibody and no positivity was observed. Nuclei were stained with 4′,6-diamidino-2-phenylinolde (1:2000). The samples were observed under Leica TCS SP2, A0BS confocal microscope. Images were taken at 260 nm intervals (∼63 magnification) (23).

**Plasmids and cell transfection**

The plasmids Jxtm1-, 2- and 3-green fluorescent protein (GFP) and Tkd1-GFP were from D.L.Rimm (Yale University, New Haven, CT). Jxtm1-, 2- and 3-GFP were cut with KpnI/BglII, blunted with Klenow polymerase and subcloned in pSG424 containing Gal4 DNA-binding domain (Gal4BD) (from S.Fields, University of Washington, Seattle, WA). For subcloning, pSG424 has been cut with KpnI/BglII, pMOG-Met (from L.Schmidt, National Cancer Institute, Frederick, MD), containing full-length Met, was cut with EcoRI and subcloned in pSG424 with EcoRI. Then, we obtained Jxtm1, Jxtm2, Jxtm3 and Met plasmids, in which entire Met and the fragments were fused with Gal4BD. The cells in 24-multiwell plates, were co-transfected with 200 ng of each plasmid-Gal4BD and of G5-38HIV-Luc (Gal4Luc) (from G.Napolitano, Mount Sinai Hospital, Toronto, Canada) or ASCR (from W.Horne, Yale University, New Haven, CT) expression vector. As internal control, the reporter plasmid encoding Renilla luciferase (40 ng per well) was co-transfected, and Firefly/Renilla luciferase activity ratios were calculated by the software. The transfections were performed for 48 h using Fugene 6 (Roche Applied Science, Mannheim, Germany) (23).

**Immunoprecipitation and western blot assays**

The cells exposed or not to HGF were pretreated for 4 h with 10 μM LLNL and used for total cell extracts, prepared in radio immunoprecipitation assay (RIPA) buffer, and for nuclear and cytosol extracts (25,26). Total, cytosol and nuclear extracts (1 mg of protein) were immunoprecipitated using 6.5 μg of anti-Met antibody (27). Before immunoprecipitation of nuclear extracts, the samples were diluted with lysis buffer (50 mM Tris–HCl, pH 7.4/1% Nonidet-P40/1 mM ethylenediaminetetraacetic acid/150 mM NaCl/1 mM Na3VO4) supplemented with proteinase inhibitors. Some cells in T25 flasks, transfected or not with 2.5 μg WWOX expression vector, were used for nuclear protein extraction or were treated with cycloheximide (100 μg/ml) for various times and used for total cell extract (18). Total protein extracts (corresponding to 100 or 300 μg of protein) from cells transfected or not with WWOX were used for western blots. Immunoblots were performed with anti-Met (1:200), anti-phospho-Met (Tyr1349) and (Tyr1234/35) (1:1000), anti-phospho-pyruvate kinase (3 μg/ml), anti-Akt (1 μg/ml), anti-phospho-Akt (1:1000), anti-YAP (1:1000) or anti-phosphoYAP (1:1000) antibody. To confirm equal loading, the membranes were immunoblotted with anti-vinculin antibody. Immunoreactive bands were evaluated by densitometric analysis after reaction with enhanced chemiluminescence kit (ECL) or ECL plus (GE Healthcare, Milan, Italy).

**Fluorescence microscopy**

The cells on coverslips were transfected with Jxtm1-GFP, Jxtm2-GFP, Jxtm3-GFP, Tkd1-GFP or Met-GFP plasmids for 24 h. To prepare Met-GFP, pMOG-Met was cut with EcoRI and subcloned in PEGFP-N1 with EcoRI. The cells were fixed with 4% paraformaldehyde and nuclei stained with 4′,6-diamidino-2-phenylinolde. The images were collected at ×400 magnification under fluorescence microscope (28).

**Matrigel invasion assay**

Matrigel invasion chambers from BD Biocoat Cellware were used (Becton Dickinson Labware, Bedford, MA). Cells transfected with WWOX or ΔAkt expression vector, resuspended in medium with 0.1% FBS, were added (8 × 10⁴ per well) to the upper chambers and allowed to invade through Matrigel. Also, the lower chambers contained 0.1% FBS medium. Cells on the lower side of the membrane were stained (10).

**RNA interference**

Cells were transfected with 90 nM small interfering RNA (siRNA) (control or YAP) and, when indicated, co-transfected with Jxtm3-Gal4BD and Gal4Luc expression vector using Lipofectamine 2000 (Invitrogen, Milan, Italy). All transfections were performed for 48 h. The harvested cells were lysed in urea buffer (8 M urea, 0.1 M NaH2PO4, 0.01 M Tris, pH 8, and protease inhibitors) to obtain proteins for western blotting analysis or were processed to evaluate luciferase activity (10). siRNA/YAP oligonucleotide sequences are those of Basu et al. (29).

**Statistical analysis**

Luciferase activity and cell counts were analyzed by analysis of variance, with P < 0.05 considered significant. Differences from controls were evaluated on original experimental data.

**Results**

**Nuclear localization of Met in MDA-MB231 cells was constitutive and independent of HGF**

Studies were performed in MDA-MB231 highly invasive breast carcinoma cells to evaluate the possible existence of a nuclear constitutively active Met-signaling pathway. Low invasive MCF-7 cells were used for comparison (10,20,29).

As shown in Figure 1A, in MDA-MB231 but not in MCF-7 cells, Met was remarkably localized in the nucleus showing a punctate pattern, both in the presence and the absence of HGF. To put in evidence these data, we show a high resolution image of MDA-MB231-un-treated cells (Figure 1A, H). Met was found in the nucleoplasm in non-membranous environment, as reported for other RTKs (15). In both cell lines, Met signal was also present in plasma membrane and cytosol (26). The treatment with HGF for 30 min seemed to cause scattering mostly in MDA-MB231 cells, with Met relocalization at focal adhesion level (Figure 1A).

To give a more quantitative evaluation, western blot analysis of Met protein levels was performed using total cell extracts. The levels of
Met precursor (170 kDa) and mature protein (145 kDa corresponding to the β-chain of Met heterodimer) were 20-fold higher in MDA-MB231 than in MCF-7 cells (Figure 1B). In MDA-MB231 cells, both tyrosine 1349, at the C-terminal regulatory portion, and tyrosines 1234/35, at the catalytic site, were constitutively phosphorylated suggesting that Met was active. The present findings indicate that Met overexpressed in MDA-MB231 cells might be implicated in its own phosphorylative activation independently of ligand binding. In agreement with previous data (10), HGF also increased cellular Met tyrosine phosphorylation at 30 min in the two cell lines used (data not shown).

Then, we verified whether nuclear Met was the full-size receptor or a portion of the β-chain at the CTF because we used a specific polyclonal antibody for the cytoplasmic domain in Met immunoprecipitation experiments (Figure 2A). Only in MDA-MB231 cells, treated or untreated with HGF, we observed a band at 60 kDa in nuclear (N) and total (T) extracts, as well as the 145 kDa band using T extracts. In T extracts of MCF-7 cells, the 145 kDa band was faint. The purity of nuclear preparations was confirmed using nuclear (histone H3) and non-nuclear (α-tubulin) markers (data not shown) (30). A Met fragment in the nuclei has been shown in human embryonic kidney cells HEK293 and some kinds of tumor cell lines (26). To obtain more information on the possible functional role of the 60 kDa Met fragment, nuclear immunoprecipitates with anti-Met antibody were immunoblotted with antibodies for Met phosphotyrosines 1349 and 1234/35. In MDA-MB231 cells, the 60 kDa band was phosphorylated on all tyrosines indicating the possible functionality of the receptor fragment without HGF stimulus. No immunoreactivity was found in MCF-7 cells with the antibodies used (Figure 2B). All these cells were treated with LLNL, a specific proteasome inhibitor, to prevent Met degradation (16,26).

To evaluate the possible role of c-Src tyrosine kinase in the control of nuclear Met activity, in a first series of experiments, we analyzed by confocal microscopy whether Met and phospho-c-Src (pSrc) co-localized in the nuclei. The images were taken at the apical sections, showing principally cell nuclei (Figure 2C). In MDA-MB231 cells exposed to HGF for 30 min, in respect to starved (−HGF) cells, we observed that nucleoplasmic punctate Met signal was remarkable and unmodified and did not co-localize with pSrc (merge image), while at perinuclear level Met diffusely increased with a pattern largely different from that of pSrc signal. In 30 min HGF-treated MCF-7 cells, compared with starved cells, Met and pSrc increased in the perinuclear compartment with a substantial co-localization. Met signal did not appear in the nucleus.

These experiments show that Met was inside the nuclei only in MDA-MB231 cells, independently of HGF, and apparently did not interact with pSrc.

Study of the transactivating activity of β-chain fragments of Met

The region of the cytoplasmic domain (C-terminal), possibly present in the nucleus of MDA-MB231 cells, and the transactivating activity were examined. As shown in Figure 3A, we transfected full-length Met and various regions of the cytoplasmic domain, differently truncated at the N-terminal and cloned into a GFP fusion vector. The constructs Jxtm1-GFP, Jxtm2-GFP, Jxtm3-GFP and Tkd1-GFP encode Met fragments truncated after tyrosine residues D972, R1004 and P1027 in the Jxtm region and after tyrosine kinase domain beginning at I1084, respectively (26). Jxtm1-, 2-, 3-GFP accumulated in the nuclei of both cell lines. Tkd1-GFP construct appeared non-specific in its localization, and Met-GFP principally localized outside the nuclei.

Fig. 1. Met in MDA-MB231 and MCF-7 cells. (A) Representative laser scanning images of basal confocal sections of cells treated or untreated with HGF for 30 min. Size bars appear in the upper left panels for MDA-MB231 and MCF-7 cells and correspond to 50 μm. Met (magenta) and nuclei (blue). H, high-resolution images of MDA-MB231 cells untreated with HGF. Size bar, 240 μm. The experiments were performed in triplicate. (B) Representative western blots (100 μg of total proteins) for Met and the tyrosine phosphorylated (pMet) forms are shown. The 170 kDa band corresponds to the Met precursor and the 145 kDa band to the mature form (β-chain). Immunoblot with anti-vinculin antibody was done for normalization. The experiments were performed three times with similar results.
To assess the transactivating activity of the various fragments, which localized in the nuclei, we measured the luciferase activity of Gal4-transactivation system (31), in response to Gal4 DNA-binding domain fused to Jxtm1, 2, 3 and to Met full length. These constructs have been cloned in our laboratory (Figure 3B). The Jxtm1, 2 and 3 constructs caused transactivation of Gal4Luc in MDA-MB231 but not in MCF-7 cells, while Met plasmid was inactive in our experimental conditions. HGF did not modify Jxtm-construct activities in either cell line (data not shown). The absolute value for Gal4Luc Firefly/Renilla luciferase activity ratio was 6 $\times$ 10$^{-1}$ in both MDA-MB231 and MCF-7 cells.

Thus, the Jxtm1, 2 and 3 entered in the nuclei of both the breast carcinoma cells studied, but showed transactivating activity only in MDA-MB231 cells.

**Regulation of Jxtm3 transactivating activity**

To understand the regulatory mechanisms involved in Jxtm3 trans-activating activity, we studied the effect of the expression vectors for Wwox and YAPs that might act as transcription factor co-repressor and co-activator, respectively, depending also on the cell line. We observed that endogenous Wwox and YAPs, included the phosphorylated forms, were oppositely expressed in MCF-7 and MDA-MB231 cells. As YAPs, the levels of Akt and phosphoAkt (pAkt) were higher in MDA-MB231 than in MCF-7 cells. (Figure 4A).

As shown in Figure 4B, YAPsiRNA transfection for 48 h completely reduced endogenous levels of YAPs in MDA-MB231 cells. Control siRNA did not modify Jxtm-construct activities in either cell line (data not shown). The absolute value for Gal4Luc Firefly/Renilla luciferase activity ratio was 6 $\times$ 10$^{-1}$ in both MDA-MB231 and MCF-7 cells.

Thus, the Jxtm1, 2 and 3 entered in the nuclei of both the breast carcinoma cells studied, but showed transactivating activity only in MDA-MB231 cells.

Figure 4C reports that in MDA-MB231 cells, characterized by Jxtm3-Gal4BD activated by endogenously elevated YAPs, no changes of luciferase activity were observed after YAP1, YAP2 or YAP2-S127A expression vector co-transfection. YAP2-S127A is an unphosphorylatable form mutated at Ser127, that seems to increase the localization of YAP in the nucleus, with complex mechanisms not completely clarified (24). In MCF-7 cells, the expression vectors for YAP1, YAP2 or YAP2-S127A enhanced Jxtm3 transactivating activity. It is worth noting that we used the Jxtm3 construct entering the nuclei of MCF-7 cells: it was activated by exogenous YAP expression vectors while being probably insensitive to endogenous Wwox-mediated relocalization. However, it is possible that endogenous Wwox can retain cellular full-length Met in the cytosol. Figure 4C also shows that c-Src expression vector (Srcwt) and the dominant negative (DSrc) did not change Jxtm3 transactivating activity in both cell lines. Nuclear Met activity was probably independent of phosphorylation via active c-Src in MDA-MB231 cells, in agreement with our confocal data showing that Met and pSrc signals did not co-localize in the nuclei. Srcwt, the expression vector for c-Src, produces the protein and the phosphorylated form depending on cell conditions. We found pSrc in our Srcwt-transfected cell lines (23,32).

As shown in Figure 4D, in MDA-MB231 cells the constitutive transactivating activity of Jxtm3 was decreased (55%) by WWOX expression vector co-transfection, while YAP2 gave an inhibitory effect only in the presence of the dominant negative of Akt (D Akt). Because D Akt alone impaired Jxtm3 activity, we suggest that D Akt might prevent YAP2 endogenous function. It is known that Akt phosphorylates YAPs (29). In MCF-7 cells, the stimulatory effect of YAP2 on Jxtm3 was prevented by D Akt.

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**Fig. 2.** Nuclear localization of a Met fragment in MDA-MB231 cells was independent of HGF. Representative immunoprecipitates (1 mg of protein) (A) of total (T), cytosolic (C) and nuclear (N) extracts and (B) of nuclear extracts from cells pretreated with 10 $\mu$M LLNL for 4 h and exposed or not to HGF during the last 30 min. Anti-Met antibody was used for immunoprecipitation (IP), and the antibodies indicated were used for blotting (IB). The 60 kDa band corresponded to Met-CTF fragment. The experiments have been repeated three times. (C) Representative laser scanning images of apical confocal sections of cells treated or untreated with HGF for 30 min. Size bars appear in the upper left panels for MDA-MB231 and MCF-7 cells and correspond to 50 $\mu$m. Met (magenta), pSrc (green) and nuclei (blue). The experiments were performed in triplicate with similar results.
Our data indicate that exogenous Wwox functioned as co-repressor in MDA-MB231 cells, devoid of endogenous Wwox and with active Jxtm3 maintained by YAPs. Exogenous YAPs were co-activators in cells devoid of endogenous YAP proteins and with constitutively inactive Met fragments as MCF-7 cells.

Wwox regulates Met stability and nuclear translocation of 60 kDa fragment, possibly relevant for MDA-MB231 cell invasiveness

We next examined whether Wwox expression might affect full-length Met stability in MDA-MB231 cells (Figure 5A). Transfection of WWOX expression vector, that increased Wwox protein level at 48 h (time 0) and for all the observation period, strongly slowed down the degradation of full-length Met (145 kDa) and the precursor (170 kDa), studied in the presence of the protein synthesis inhibitor cycloheximide. In contrast, full-length Met protein level was constant for all the observation period in MCF-7 cycloheximide-treated cells probably because of the highly expressed endogenous Wwox.

Then, in MDA-MB231 cells under the same experimental conditions of WWOX transfection that similarly increased Wwox protein level at 24 and 48 h, we studied Met-CTF nuclear translocation.
WWOX transfection prevented the nuclear accumulation of the 60 kDa Met fragment, and thus the constitutive Met activity was probably impaired as shown by the absence of phosphotyrosine signal corresponding to Met fragment in 48 h WWOX transfected cells (Figure 5B).

The functional role of Wwox was examined with the Matrigel invasion assay system, which is considered an in vitro model of metastasis (10,33) (Figure 5C). We observed that 48 h WWOX transfection reduced (-35%) spontaneous MDA-MB231 cell migration. D\textsubscript{Akt} transfection gave a similar inhibitory effect (-40%), probably by affecting endogenous YAP co-activator function regulating Met activity.

In conclusion, the absence of Wwox protein expression in MDA-MB231 cells seemed to be implicated in nuclear translocation of an active Met fragment, maintaining the transduction signaling for spontaneous invasiveness of MDA-MB231 cells.

Discussion

We report for the first time that the presence of active Met-CTF fragments in the cell nuclei was related with breast carcinoma aggressiveness and depended on WWOX expression. Only in invasive/metastatic MDA-MB231 breast carcinoma cells, nuclear Met seemed to be constitutively active in regulating gene transcription. First, we observed that Met-CTFs were tyrosine phosphorylated at regulatory and catalytic domains without HGF stimulus. Second, transfected constructs containing Jxtm fragments, differently truncated at N-terminal domain of Met, entered the nuclei and showed transactivating activity. Thus, Met fragments probably played a role as transcription factors in the nucleus of MDA-MB231 cells also in the absence of HGF stimulus. The difference with low invasive MCF-7 cells was evident, because the latter did not show intranuclear Met and the transfected constructs containing Jxtm fragments were inactive even if exposed to HGF.

The transport of RTKs (Met and Erb-B4) to the nucleus begins with different mechanisms of internalization also depending on the cell type. In the liver cell line SkHep1, Met is excluded from the nucleus but rapidly moves to the nucleus upon stimulation with HGF depending on Gab1 and importin \( \beta_1 \) (30). Our observations in MDA-MB231 tumor cells indicate that invasive/metastatic cells might take advantage from endogenously constitutive activation of Met signaling in the...
The aggressive cells probably become independent from the environmental HGF for certain critical steps of metastatization. For Erb-B4, extensive reviews have been published (15,31,34).

The functional role of nuclear Met was related to the spontaneous migration of MDA-MB231 cells, possibly by regulating the transcription of genes for invasiveness. MDA-MB231 cells were practically devoid of Wwox tumor suppressor, explaining various aspects of nuclear Met-CTF activity and localization and cell behavior. In fact, the re-establishment of Wwox expression in MDA-MB231 cells partly reduced migration, probably blocking the signaling downstream of Met-CTF transcription factors. We suggest that the mechanistic basis of exogenous Wwox action was the impairment of Met-CTF nuclear localization and the inhibition of its transactivating activity. Also, Wwox tumor suppressor function was related to the maintenance of full-length Met stability, as occurred indeed in MCF-7 cells endowed of Wwox.

Noteworthy, full-length Wwox and newly synthesized Met are present in Golgi apparatus. Receptor-bearing endosomes fuse with Golgi, are retrograde transported to the endoplasmic reticulum, and RTKs, extracted by endoplasmic reticulum-associated degradation (ERAD) system, are not degraded but transported to the nucleus (15,20,35). In this system, we can envision a model for regulating Met stability/fragmentation/nuclear transport, related to tumor progression, in view of the role played by Wwox (20). Our data are in agreement with the findings that WWOX gene is altered in many human cancers, including breast cancer, and that the expression of WWOX appears inversely correlated with tumor aggressiveness (18). The WWOX gene spans a fragile genomic region that is frequently affected by hemizygous loss in the vast majority of breast cancer cell lines and primary tumors and sometimes by homozygous deletions (20). Epigenetic mechanisms such as methylation may also affect WWOX expression (36).

Akt activity may regulate transcription factor activities and YAP functions (17,24,29). We observed that in MDA-MB231 cells Akt was involved in constitutive Met-CTF transactivating activity, probably permitting the co-activator function of YAPs and participated, therefore, in the spontaneous migration of MDA-MB231 cells. We cannot exclude that Met-CTF alone, due to Wwox absence, might move to the nucleus where YAPs already exist, in complex with other transcription factors, and are probably to be regulated by Akt (24,37).

Fig. 5. Wwox protein expression in MDA-MB231 cells regulated Met full-length stability, intranuclear localization of Met-CTF fragments and invasiveness. (A) Cells transfected or not with WWOX expression vector were treated with cycloheximide (CHX) at time 0, i.e. 48 h after WWOX transfection. C, control non-transfected cells. Aliquots of 100 and 300 μg of total proteins were used for western blots of MDA-MB231 and MCF-7 cells, respectively. Immunoblot with anti-vinculin antibody was done for normalization. The experiments have been performed in triplicate with similar results. (B) Representative immunoprecipitates (1 mg of protein) of nuclear extracts from cells transfected or not with WWOX expression vector and treated with 10 μM LLNL. The anti-Met antibody was used for immunoprecipitation (IP), and anti-Met or anti-phosphotyrosine (pTyr) antibodies were used for blotting (IB). The experiments have been repeated three times. (C) 24 h transfected (WWOX or Akt expression vector) and control (non-transfected, C) cells were used for Matrigel invasion assay (22 h), adding culture medium with 0.1% FBS to upper and lower chambers. To estimate invasion, we counted (×200 magnification) the invading cells on the lower side of the membrane after staining. Representative images are shown, and the numbers at the bottom are the mean ± SE of the counts of ten selected fields for six independent experiments. *P < 0.05 versus control value.
suggest that in MDA-MB231 cells, endogenously elevated and phosphorylated YAPs probably intervened as co-activators in nuclear Met transactivating activity, because of its impairment by YAP knocking down. In MDA-MB231 cells, Jxtm3 transactivating activity was, however, unaffected by exogenous YAPs at difference with MCF-7 cells. In the latter, exogenous YAP-induced Jxtm3 activity was antagonized by Wwox, as reported for Erb-B4 (17).

Nuclear Met-CTF was tyrosine phosphorylated in MDA-MB231 cells, but c-Src did not seem implicated for the following reasons: the Met/pSrc signal did not co-localize in the nuclei, also in the presence of HGF. Srcwt did not increase transactivating activity and ΔSrc was ineffective. In MCF-7 cells, the plasma membrane-Met seemed to co-translocate with pSrc and localized in the perinuclear compartment after HGF treatment, possibly contributing to a continuous delivery of signals after being endocytosed (16).

The functional importance and the mechanism of RTK-CTF translocation to the nucleus are not clear, but the extension of the phenomenon to various kinds of receptors indicates an important role of this localization. For Erb-B1, addition of its cognate ligand causes translocation of the ligand–receptor complex to nucleus. For Met, it has been suggested that regulated intramembrane proteolysis (RIP) leads to nuclear localization of the cytoplasmic domain, independent of ligand binding. The Met-CTF fragment is not derived by alternative splicing of the messenger (26). We propose a signaling model integrating possible connections between Met/Wwox/YAP/Akt in the two breast carcinoma cell lines (Figure 6).

In conclusion, our findings add new mechanistic aspects regarding the role of Wwox endogenous expression in low invasive, in respect to highly invasive/metastatic cells lacking Wwox, as regards Met nuclear localization and transcription factor function. This molecular mechanism might correlate the clinical significance of Wwox to differential prognostic significance of cell membrane associated full-length Met and the nucleus-associated CTF (19). Met multifunctional docking site is important for the control of multiple functions connected with invasive growth such as motility, proliferation/survival and apoptosis (2,38). The constitutive activation of nuclear Met-signaling pathway seemed to be determined at genetic or epigenetic (methylation) level of WWOX (20,36) and participated in the complex process of MDA-MB231 spontaneous migration.

**Fig. 6.** Signaling model integrating possible connections between Met/Wwox/YAP/Akt in MDA-MB231 and MCF-7 cells.

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**References**


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