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Mol Cancer Res Published OnlineFirst August 30, 2012.

Published OnlineFirst on August 30, 2012; DOI:10.1158/1541-7786.MCR-12-0312

Angiogenesis, Metastasis, and the Cellular Microenvironment

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Copper Modulates Zinc Metalloproteinase-Dependent Ectodomain Shedding of Key Signaling and Adhesion Proteins and Promotes the Invasion of Prostate Cancer Epithelial Cells

Catherine A. Parr-Sturgess[1], Claire L. Tinker[1], Claire A. Hart[2], Michael D. Brown[2], Noel W. Clarke[2], and Edward T. Parkin[1]

Abstract

A disintegrin and metalloproteinases (ADAMs) and matrix metalloproteinases (MMPs) are zinc metalloproteinases (ZMPs) that catalyze the "ectodomain shedding" of a range of cell surface proteins including signaling and adhesion molecules. These "sheddases" are associated with the invasion and metastasis of a range of cancers. Increased serum and tumor tissue levels of copper are also observed in several cancers, although little is known about how the metal might promote disease progression at the molecular level. In the current study, we investigated whether copper might regulate the ectodomain shedding of two key cell surface proteins implicated in the invasion and metastasis of prostate cancer, the Notch ligand Jagged1 and the adhesion molecule E-cadherin, and whether the metal was able to influence the invasion of the prostate cancer epithelial cell line PC3. Physiological copper concentrations stimulated the ZMP-mediated proteolysis of Jagged1 and E-cadherin in cell culture models, whereas other divalent metals had no effect. Copper-mediated Jagged1 proteolysis was also observed following the pretreatment of cells with cycloheximide and in a cell-free membrane system, indicating a posttranslational mechanism of sheddase activation. Finally, the concentrations of copper that stimulated ZMP-mediated protein shedding also enhanced PC3 invasion; an effect that could be negated using a sheddase inhibitor or copper chelators. Collectively, these data implicate copper as an important factor in promoting prostate cancer cell invasion and indicate that the selective posttranslational activation of ZMP-mediated protein shedding might play a role in this process. *Mol Cancer Res; 1–12. ©2012 AACR.*

Introduction

The proteolytic cleavage of proteins within their juxtamembrane region and subsequent ectodomain release, a process known as "ectodomain shedding," represents a common molecular mechanism for regulating the biological activity of a range of cell surface proteins including signaling and adhesion molecules (1). Two classes of zinc metalloproteinases (ZMPs) have been implicated as "sheddases;" the a disintegrin and metalloproteinases (ADAMs) and matrix metalloproteinases (MMPs) (2, 3). Ectodomain shedding constitutes the rate-limiting step in a group of signaling pathways involving the regulated intramembrane proteolysis (RIP) of cell surface membrane proteins (4), the original paradigm for which was Notch signaling (5). RIP is initiated by sheddase cleavage of the substrate protein generating a soluble ectodomain and a residual membrane-associated C-terminal fragment (CTF) which can be further processed by a presenilin-dependent γ-secretase complex (6). This second proteolytic event liberates a soluble intracellular domain (ICD), which, in the case of certain proteins, is capable of nuclear translocation and subsequent transcriptional regulation (7). Given the functionality of the proteins subject to ectodomain shedding and RIP, it is perhaps not surprising that ADAMs and MMPs have been linked to the invasion and metastasis of many different types of cancer (2, 3).

Jagged1 is one of five known mammalian Notch ligands, which like its receptor, has been linked to the progression of various types of cancer (8). LaVoie and Selkoe (2003) (9) showed that Jagged1 was subject to ectodomain shedding, being cleaved first by an ADAM-like activity to release a soluble fragment (sJag) with the residual Jagged1 C-terminal fragment (JCTF) being cleaved by a γ-secretase activity generating a transcriptionally active Jagged1 intracellular

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Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

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doi: 10.1158/1541-7786.MCR-12-0312

Published OnlineFirst on August 30, 2012; DOI:10.1158/1541-7786.MCR-12-0312

domain (JICD). Work in our laboratory (10) implicated ADAM17 as the key enzyme responsible for the constitutive shedding of Jagged1.

The involvement of E-cadherin in cell–cell interaction is well established in embryonic development, organ morphogenesis, tissue integrity, and wound healing (11) and the protein is considered to function as a metastasis suppressor due to its inhibition of cancer cell migration and invasion (12). E-cadherin is subject to ectodomain shedding by several proteinases including MMP3, MMP7, ADAM10, and ADAM15 (13). Cleavage of mature E-cadherin results in the release of soluble fragments of the extracellular domain (sE-CAD) and increased invasive behavior of cells (13). The residual membrane-associated E-cadherin fragment generated by metalloproteinase cleavage can be further processed by a γ-secretase activity generating a soluble ICD capable of nuclear translocation and transcriptional regulation (14).

Both Jagged1 and E-cadherin have been implicated in the pathogenesis of prostate cancer. Downregulation of the former protein in a range of prostate cancer epithelial cells (PECs) inhibits cell proliferation and induces growth arrest in the S phase of the cell cycle (15). Jagged1 expression in PECs also increases the expression of antiapoptotic proteins (16). Expression of the ligand is significantly higher in metastatic prostate cancer as compared with benign or localized disease, further indicating that the protein may be linked to the growth, metastasis and progression of prostate tumors (17). Loss of E-cadherin expression correlates with the invasive phenotype of PECs (18, 19) and reduced expression correlates inversely with tumor grade. Cleavage of full-length E-cadherin has been linked to the malignant progression of adenocarcinomas including prostate cancer (20, 21) and an 80 kDa soluble fragment of the protein was almost exclusively observed in the neoplastic aspect of prostate cancer (22).

A handful of reports exist showing increased copper levels in the whole blood, plasma, or serum of patients with prostate cancer (23–25). However, while the proangiogenic effect of the metal is well known (26) little is known about the effects, if any, of copper on the invasion and metastasis of cancerous cells. In the current report we have studied Jagged1 and E-cadherin, as model sheddase substrates and as key signaling and adhesion molecules implicated in prostate cancer, to determine whether copper could stimulate their proteolysis and the invasion of PECs. Our results have shown that physiologically relevant copper concentrations can stimulate ZMP-mediated proteolysis of the two proteins and, in the case of Jagged1 at least, the subsequent γ-secretase–mediated generation of a soluble ICD. Mechanistically, our results also indicate that copper acts at the posttranslational level in relation to sheddase activation. We also show that the invasion of PECs was stimulated by exogenous copper and that this effect could be ablated using a sheddase inhibitor. Furthermore, the chelation of endogenous copper completely abolished the stimulatory effect of bone marrow stroma (BMS) on PEC invasion. Collectively, these data implicate copper as an important factor in promoting PEC invasion and indicate that the selective post-translational stimulation of ZMP-mediated protein shedding might play a role in this process.

Materials and Methods

Materials

The human full-length Jagged1 plasmid, pIREShyg-Jagged1, was synthesized by Epoch Biolabs and has been fully characterized previously (10). Anti-Jagged1 ectodomain and anti–E-cadherin ectodomain polyclonal antibodies were from R&D Systems Europe Ltd., and anti-Jagged1 C-terminal polyclonal antibody was from Santa Cruz Biotechnology Inc. Anti-actin monoclonal antibody and anti-amyloid precursor protein (APP) C-terminal polyclonal antibody were from Sigma-Aldrich Company Ltd.. Anti-APP 6E10 monoclonal antibody was from Cambridge Bioscience Ltd.. All other materials, unless otherwise stated, were purchased from Sigma-Aldrich Company Ltd.

Cell culture and viability assays

Cells were purchased from the American Type Culture Collection and used within 6 months of identity verification by the Paterson Institute for Cancer Research (Manchester, United Kingdom) tissue typing service. All cell culture reagents were purchased from Lonza Ltd.. HEK-Jagged1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 25 mmol/L glucose, 4 mmol/L L-glutamine, and 10% (v/v) FBS. PC3 cells were cultured in Ham's F12 medium supplemented with 7% (v/v) FBS and 2 mmol/L L-glutamine. All cells were maintained at 37°C in 5% CO_2 in air. Cell viability was assessed by monitoring the reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) using the Cell Titer 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions or by monitoring trypan blue dye exclusion.

Treatment of cells and protein extraction

For the study of protein shedding, cells were grown to confluence in 75 cm^3 flasks and rinsed twice *in situ* with reduced serum medium (10 mL; OptiMEM; Invitrogen). A fresh 10 mL of OptiMEM was then conditioned on cells for the indicated periods. Copper and other metals were added to the final OptiMEM incubation as free salts or as glycine complexes generated by premixing the metal salts with a 10-fold molar excess of glycine. Ilomastat was added to cells at the indicated concentrations alongside control flasks treated with an equal volume of dimethyl sulfoxide (DMSO) vehicle. The copper chelators bathocuproine and D-penicillamine were incorporated in the final OptiMEM incubation where stated at 200 μmol/L final concentrations. Media for immunoblot analyses were harvested, centrifuged at 10,000 × *g* for 10 minutes to remove cell debris, and concentrated 50-fold using Vivaspin 6 centrifugal concentrators (Sartorius, Epsom). For analysis of cell-associated proteins, cells were washed with PBS (20 mmol/L Na_2HPO_4, 2 mmol/L NaH_2PO_4, 0.15 mol/L NaCl, pH 7.4) and scraped from the

Published OnlineFirst on August 30, 2012; DOI:10.1158/1541-7786.MCR-12-0312

flasks into fresh PBS (10 mL). Following centrifugation at 500 × *g* for 5 minutes, cell pellets were lysed in 100 mmol/L Tris, 150 mmol/L NaCl, 1% (v/v) Triton X-100, 0.1% (v/v) Nonidet P40, 10 mmol/L 1,10-phenanthroline, pH 7.4.

Cell-free shedding assay

HEK-Jagged1 cells were disrupted in buffer A (10 mmol/L Tris, 150 mmol/L NaCl, 0.1 mmol/L $ZnCl_2$, pH 7.4). Samples were then centrifuged at 20,000 × *g* (20 minutes) before recentrifuging the supernatant at 100,000 × *g* (90 minutes). The membrane pellet was resuspended in buffer A and used immediately for shedding assays. Aliquots (50 μL) of the resuspended membranes were preincubated on ice for 20 minutes in the absence/presence of ilomastat (20 μmol/L) or DMSO vehicle alone before the addition of free copper (30 μmol/L). The samples were then incubated immediately at 37°C for 1 hour before making them up to 200 μL with buffer A containing 1% (v/v) Triton X-114 (precondensed), and incubating at 4°C for 10 minutes. Samples were then vortexed, incubated at 30°C for 10 minutes and then centrifuged at 3,000 × *g* for 3 minutes. The resulting detergent-rich phase was made up to the same volume as the detergent-poor phase by the addition of buffer A.

Protein assay

Protein was quantified using bicinchoninic acid (27) in a microtiter plate with bovine serum albumin (BSA) as a standard.

SDS-PAGE and immunoelectrophoretic blot analysis

Samples were mixed with a half volume of reducing electrophoresis sample buffer and boiled for 3 minutes. Proteins were resolved by SDS-PAGE using 5% to 15%, 7% to 17%, or 5% to 20% polyacrylamide gradient gels and transferred to Immobilon P polyvinylidene difluoride (PVDF) membranes (28). Anti-Jagged1 C-terminal and anti-Jagged1 ectodomain antibodies were used at 1:2,000 and 1:500, respectively. Anti-actin antibody was used at 1:5,000 and the anti–E-cadherin antibody was used at 1:1,000. Finally, anti-APP 6E10 antibody and the anti-APP C-terminal antibody were used at 1:2,500 and 1:7,500, respectively. Bound antibody was detected using peroxidase-conjugated secondary antibodies (Sigma-Aldrich Company Ltd. and R&D Systems Europe Ltd.) in conjunction with enhanced chemiluminescence detection reagents (Perbio Science Ltd.).

RNA extraction and semiquantitative reverse transcription-PCR

RNA from cell samples was extracted using TRIzol (Invitrogen). Samples containing 1 μg of RNA were then subjected to total cDNA strand synthesis followed by PCR amplification using a Titanium one-step reverse transcription-PCR (RT-PCR) kit (Clontech) according to the manufacturer's instructions. The Jagged1 primer sequences were: 5′-CCTGCACTGGTGTGGGCGAG-3′ and 5′-TCAGCTGCTCCCGCACGTTG-3′ giving a PCR product of 573 bp. The E-cadherin primer sequences were: 5′-ACCACCTCCACAGCCACCGT-3′ and 5′-GTCCAGTTGGCACTCGCCCC-3′ giving a PCR product of 518 bp. The actin primer sequences were: 5′-TGAAGTGTGACGTGGACATCCG-3′ and 5′-GCTGTCACCTTCACCGTTCCAG-3′ giving a PCR product of 447 bp.

Invasion assays

FluoroBlok cell culture inserts (8 μm; BD Biosciences) coated with Matrigel diluted 1:25 with DMEM, were placed in a 24-well plate containing 1 mL of DMEM/0.1% (w/v) BSA. An endothelial barrier was formed by confluent bone marrow endothelial cells [BMEC; (29) and Supplementary Methods] cultured on top of the Matrigel within the insert (30). Inserts were then transferred to new plates containing growth medium and either tissue culture plastic (TCP) alone or human BMS. PC3 cells stably expressing GFP were preincubated with copper, ilomastat, or copper chelators at the indicated concentrations before seeding them into the inserts on the surface of the BMEC barrier. The same concentrations of these compounds were also included in medium throughout the course of the invasion assays. The invasion of the PC3-GFP cells through the Matrigel/BMEC barrier was assessed at hourly intervals or at an endpoint of 24 hours by bottom reading of fluorescence on a BMG Labtech FLUOstar OPTIMA plate reader at 488/520 nm (excitation/emission filter). This plate reader is a self-contained incubator which maintains temperature and CO_2 levels at 37°C and 5%, respectively, allowing for the continuous automated monitoring of invasion at hourly intervals without the removal of the plate from the reader.

Statistical analysis

All data are presented as the means ± SD or SEM (indicated in the figure legends). Data were subjected to statistical analysis via Student *t* test. Levels of significance are also indicated in the figure legends.

Results

Copper enhances Jagged1 RIP in HEK-Jagged1 cells

Endogenous levels of Jagged1 expression in PECs do not generally permit the detection of the minor pool of the protein processed by γ-secretase to form the JICD. Therefore, to gain a complete insight into the potential effects of copper on Jagged1 proteolysis, we initially used our previously characterized HEK-Jagged1 cell system (10).

"Exchangeable" copper in plasma bound to amino acids has been estimated at 3.6 μmol/L, whereas the total copper levels in plasma and seminal fluid are measured at 15.5 ± 9 and 5.9 ± 3.7 μmol/L, respectively (31, 32). Thus, we initially chose to employ copper concentrations of 0, 1, 10, and 50 μmol/L in both the free salt form and as a copper–glycine complex, the latter of which perhaps better approximates the presentation of the metal in biological fluids.

Initially, we examined the effect of copper on cell viability by incubating HEK-Jagged1 cells for 5 hours with the metal before conducting an MTS assay. The results (Fig. 1A) show that, regardless of whether free or glycine-complexed copper

Published OnlineFirst on August 30, 2012; DOI:10.1158/1541-7786.MCR-12-0312

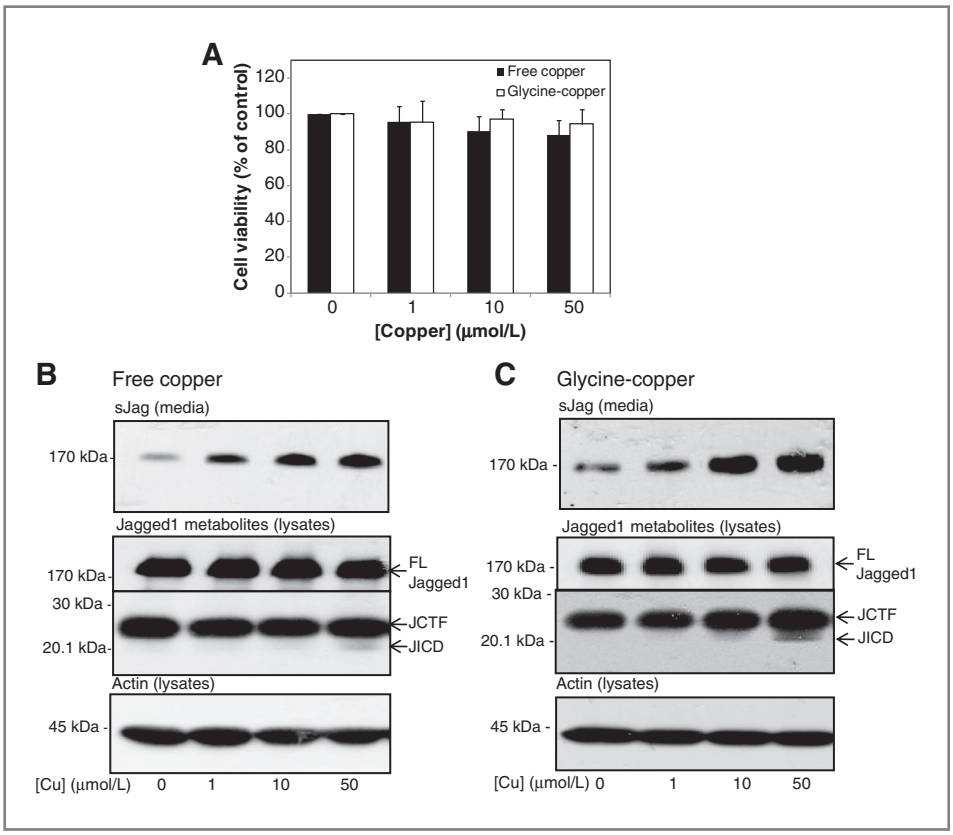

Figure 1. Copper enhances Jagged1 ectodomain shedding and RIP in HEK-Jagged1 cells. Cells were incubated for 5 hours in the presence of the free or glycine-complexed copper concentrations indicated. A, MTS assay showing a lack of cytotoxicity at any of the copper concentrations employed. B and C, the effect of free (B) and glycine-complexed (C) copper on Jagged1 proteolysis. Concentrated conditioned media were immunoblotted with anti-Jagged1 ectodomain antibody to detect sJag, whereas cell lysates were immunoblotted with anti-Jagged1 C-terminal antibody to detect full-length Jagged1, JCTF, and JICD. Lysate samples were also immunoblotted with anti-actin antibody. Results are means ± SD ($n = 3$). Unless otherwise indicated, results were not significantly different. Immunoblots are representative of 3 independent experiments.

was used, none of the metal concentrations employed were cytotoxic. However, over the same time course, both free and glycine-complexed copper identically enhanced sJag shedding into conditioned medium in a dose-dependent manner beginning at concentrations as low as 1 μmol/L (Fig. 1B and C). While levels of the full-length Jagged1 protein and the JCTF did not change at any of the copper concentrations studied, both free copper and copper–glycine enhanced the generation of the JICD at the highest copper concentration (50 μmol/L; Fig. 1B and C). In the presence of glycine-complexed copper (Fig. 1C), this latter effect was also just about detectable at the 10 μmol/L metal concentration.

Copper enhances Jagged1 RIP via a mechanism involving posttranslational sheddase activation

To shed some light on the mechanism of copper-regulated protein shedding, we first examined whether the activation of "classic" ZMP sheddases was involved by testing whether the phenomenon could be ablated by the sheddase inhibitor, ilomastat. Note that, in order to effectively visualize the stimulatory effect of copper on JICD generation, we chose to use a metal concentration of 30 μmol/L in this section of the study. The results (Fig. 2A) clearly show that, while copper enhanced sJag shedding by 2.95 ± 0.16-fold, this effect could be completely ablated by coincubation with copper and ilomastat. In fact, the level of sJag generated in the presence of copper and ilomastat was indistinguishable from that generated in the presence of the inhibitor alone. The corresponding cell lysates were then immunoblotted with anti-Jagged1 C-terminal antibody. The results (Fig. 2B) show that, as previously observed (Fig. 1), copper did not alter the cell-associated levels of either full-length Jagged1 or the JCTF but did enhance JICD levels 2.56 ± 0.52-fold; an effect which, again, was completely ablated by cotreatment of the cells with the metal and ilomastat.

Next, we examined whether the *de novo* synthesis of sheddase protein was required for copper to promote Jagged1 proteolysis. HEK-Jagged1 cells were pretreated with cycloheximide before replacing the medium with Opti-MEM with/without cycloheximide and/or copper and incubating for an additional 2 hours before examining Jagged1 shedding into the conditioned medium. The results (Fig. 2C) show that, while the overall amount of Jagged1 shed in the presence of cycloheximide was reduced (presumably due to the impaired synthesis of the protein), the fold increase in shedding following copper treatment was no different to that observed in the absence of cycloheximide. Thus, *de novo* sheddase synthesis was not required. Furthermore, we showed (Fig. 2D) that copper enhanced the ZMP-mediated shedding of Jagged1 in a cell-free membrane preparation (see Materials and Methods) further showing that neither *de novo* protein synthesis nor an intact secretory pathway was required.

Collectively, these data clearly indicate that the stimulation of Jagged1 RIP by copper involves the posttranslational activation of ZMP sheddase activity.

Published OnlineFirst on August 30, 2012; DOI:10.1158/1541-7786.MCR-12-0312

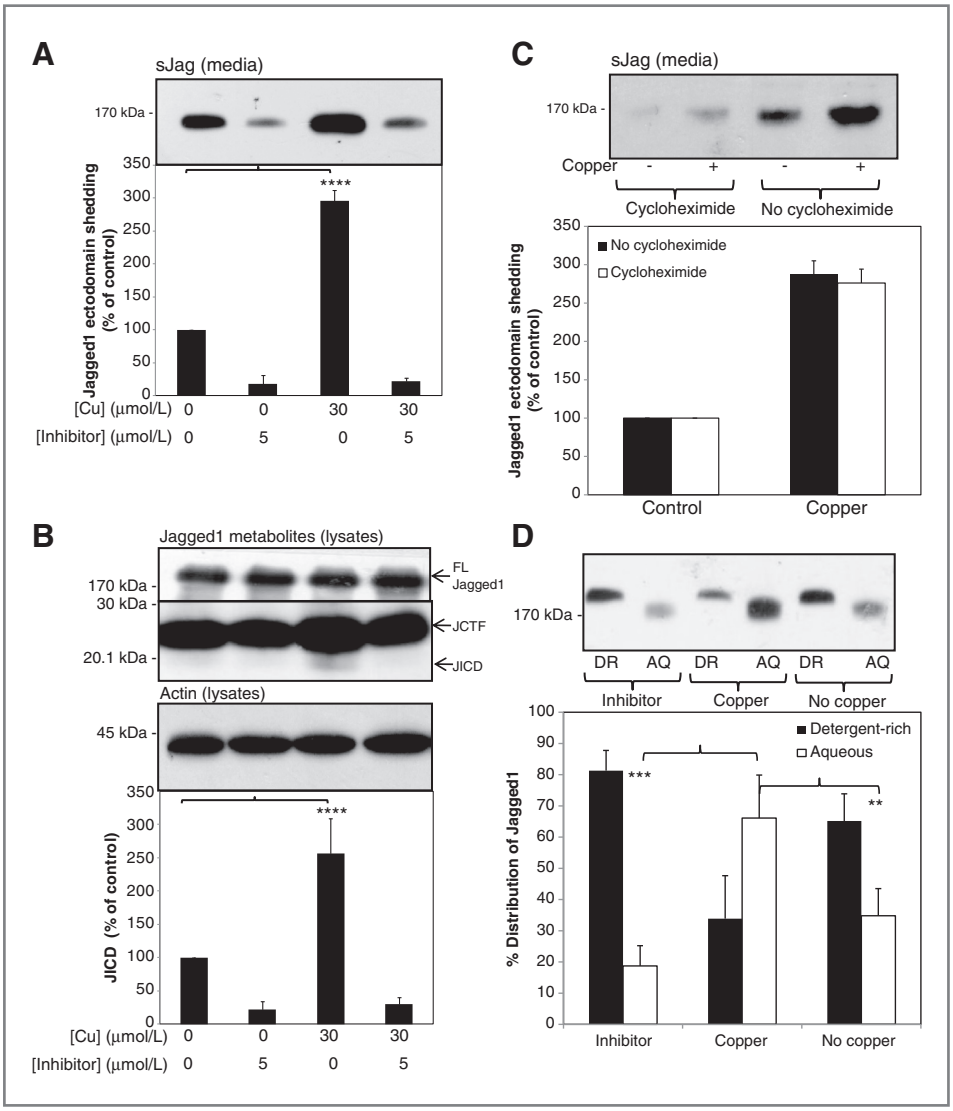

Figure 2. Copper-mediated Jagged1 proteolysis in HEK-Jagged1 cells involves the posttranslational activation of ZMP sheddase activity. A and B, cells were incubated in the absence or presence of copper (30 μmol/L) and/or the sheddase inhibitor ilomastat (5 μmol/L) for 5 hours. A, concentrated conditioned media were immunoblotted with anti-Jagged1 ectodomain antibody to detect sJag. B, cell lysates were immunoblotted with anti-Jagged1 C-terminal antibody to detect full-length Jagged1, JCTF, and JICD and with anti-actin antibody. C, HEK-Jagged1 cells were preincubated with 20 μg/mL cycloheximide for 2 hours before replacing the medium with fresh OptiMEM with/without cycoheximide and/or copper (30 μmol/L) and incubating for a further 2 hours. Conditioned medium was then harvested and immunoblotted with the anti-Jagged1 ectodomain antibody. D, membranes prepared from HEK-Jagged1 cells were preincubated on ice in the absence/presence of ilomastat (20 μmol/L) for 20 minutes before the addition of copper (30 μmol/L) and subsequent incubation at 37°C for 1 hour. The samples were then subjected to phase separation in Triton X-114 to partition full-length membrane-associated Jagged1 and hydrophilic soluble Jagged1 into the detergent-rich (DR) and aqueous (AQ) phases, respectively. The resultant phases were made up to equal volumes before resolving proteins by SDS-PAGE and immunoblotting with anti-Jagged1 ectodomain antibody. Immunoblots are representative of 3 independent experiments and were quantified by densitometric analysis. Results are means ± SD ($n = 3$). **, $P \leq 0.01$; ***, $P \leq 0.005$; ****, $P \leq 0.001$; unless otherwise indicated, results were not significantly different.

The effects elicited by copper on jagged1 proteolysis do not extend to other divalent metals

To examine whether or not the effects elicited by copper on Jagged1 proteolysis were a general phenomenon among divalent metals, we next treated HEK-Jagged1 cells with 30 μmol/L copper, iron, magnesium, zinc, calcium, or manganese for a 5-hour period and subsequently analyzed Jagged1 expression and proteolysis. The results clearly show that copper was unique among the metals studied in stimulating sJag shedding into the conditioned medium (Fig. 3A). Similarly, no metals other than copper were capable of enhancing JICD generation (Fig. 3B).

E-cadherin, but not amyloid precursor protein, shedding is enhanced by copper in a manner similar to that of jagged1

Next, to determine whether the effect of copper on ectodomain shedding was specific to Jagged1 or a general

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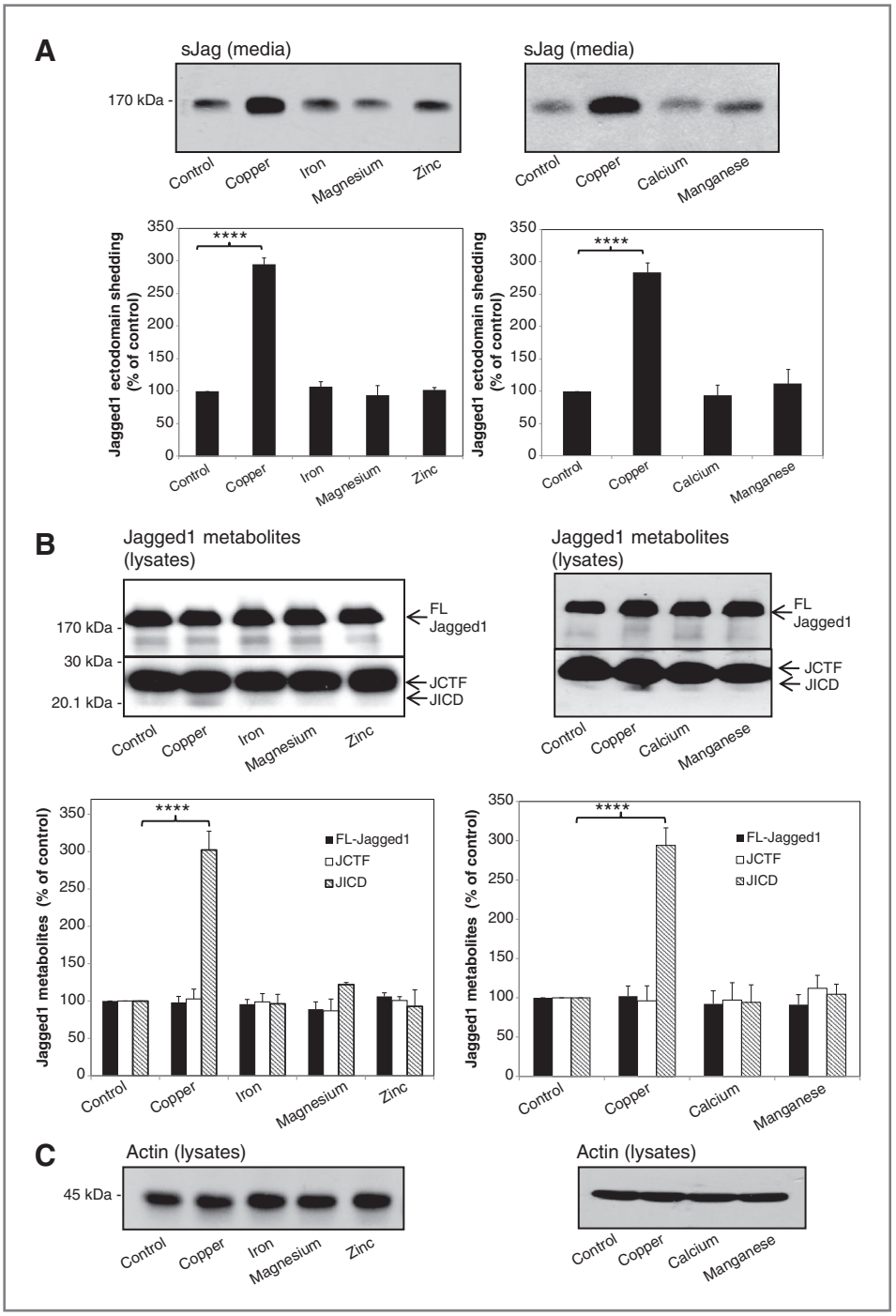

Figure 3. Metal-mediated proteolysis of Jagged1 in HEK-Jagged1 cells is specific to copper. Cells were incubated in the absence/presence of metals (30 μmol/L) for 5 hours. A, concentrated conditioned media were immunoblotted with anti-Jagged1 ectodomain antibody to detect sJag. B, cell lysates were immunoblotted with anti-Jagged1 C-terminal antibody to detect full-length Jagged1, JCTF, and JICD. C, lysate samples were immunoblotted with antiactin antibody. Immunoblots are representative of 3 independent experiments and were quantified by densitometric analysis. Results are means ± SD ($n = 3$). ****, $P \leq 0.001$; unless otherwise indicated, results were not significantly different.

phenomenon among all sheddase substrates, we examined the effect of the metal on the shedding of two endogenous substrates in HEK cells; E-cadherin and APP (13, 33). The results (Fig. 4A) show that copper (30 μmol/L) stimulated the shedding of E-cadherin by 5.12 ± 0.32-fold; an effect that could be entirely ablated by coincubation of the cells with copper and ilomastat. As was the case for Jagged1 shedding, copper stimulated E-cadherin shedding at metal concentrations as low as 1 μmol/L (data not shown). In contrast, at 30 μmol/L copper, the shedding of APP was not affected (Fig. 4B), despite the basal shedding of the protein clearly being inhibited by ilomastat. In fact, we did not observe any change in APP shedding until much higher (>200 μmol/L) metal concentrations (data not shown). The expression levels of E-cadherin and APP in cell lysates were unaffected by copper (Fig. 4C).

Collectively, these data indicate that, while the effect of copper on ZMP-mediated ectodomain shedding is not

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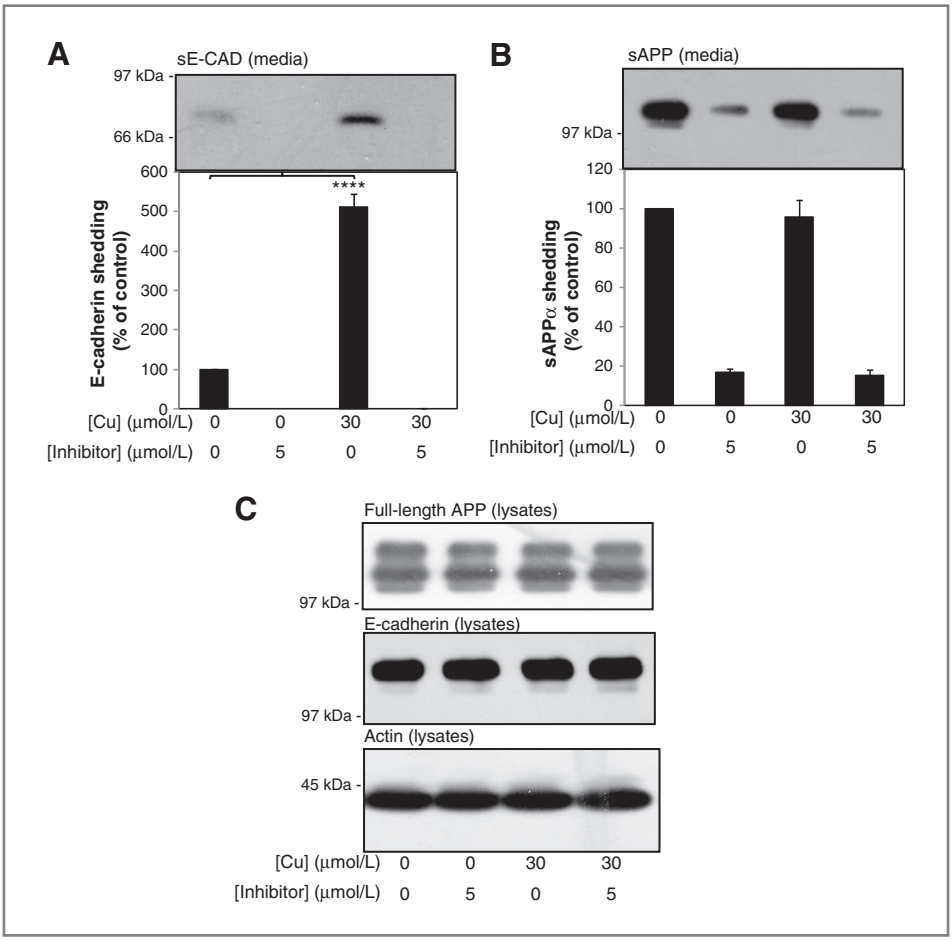

Figure 4. E-cadherin, but not APP, shedding from HEK-Jagged1 cells is enhanced by copper. Cells were incubated in the absence or presence of copper (30 μmol/L) and/or the sheddase inhibitor ilomastat (5 μmol/L) for 5 hours. A, concentrated conditioned media were immunoblotted with anti–E-cadherin ectodomain antibody to detect sE-CAD. B, concentrated conditioned media were immunoblotted with anti-APP 6E10 antibody to detect soluble APP (sAPP). C, cell lysates were immunoblotted with anti-APP C-terminal and anti–E-cadherin antibodies to detect the full-length forms of the proteins. Lysate samples were also immunoblotted with anti-actin antibody. Immunoblots are representative of 3 independent experiments and were quantified by densitometric analysis. Results are means ± SD ($n = 3$). ****, $P \leq 0.001$; unless otherwise indicated, results were not significantly different.

specific to Jagged1, the phenomenon clearly does not extend in an identical manner to all ZMP substrates.

Chelation of endogenous copper impairs the shedding of Jagged1 and E-cadherin

We next sought to determine whether shedding could be impaired through the chelation of endogenous copper. HEK-Jagged1 cells were incubated with a combination of bathocuprione and D-penicillamine (200 μmol/L) for 5 hours before analyzing Jagged1 and E-cadherin shedding. The results (Fig. 5) show that copper chelation reduced sJag (Fig. 5A) and sE-CAD (Fig. 5B) release into the conditioned medium by 53.02% ± 15.16% and 41.88% ± 16.86%, respectively. Despite this, no statistically significant increase in cell-associated levels of full-length Jagged1 or E-cadherin could be detected following chelator treatment (Fig. 5C and D, respectively). Similarly, no change in cell-associated JCTF levels was detected and, in the absence of exogenous copper, levels of JICD were below the limits of detection (Fig. 5C).

Copper promotes the ZMP-regulated ectodomain shedding of endogenous Jagged1 and E-cadherin in PC3 cells

Before examining the potential effects of copper on the invasion of PECs, it was first necessary to establish that our protein shedding results observed in HEK-Jagged1 cells translated into a suitable PEC model; specifically the bone metastatic cell line PC3. As the endogenous levels of Jagged1 in these cells were much lower than those in our HEK-Jagged1 cell system, the JICD could not be detected even at copper concentrations exceeding those, which we considered of physiological relevance (>20 μmol/L; data not shown). In the absence of an experimental rationale for extending copper concentrations beyond physiologically relevant limits, we examined the effects of 1 and 10 μmol/L metal concentrations on the ectodomain shedding of endogenous Jagged1 and E-cadherin in PC3 cells. Neither of these two copper concentrations were cytotoxic (Fig. 6A) but did enhance sJag release into conditioned medium by 3.42 ± 0.61- and 3.67 ± 0.66-fold, respectively (Fig. 6B). Similarly, 1 and 10 μmol/L copper treatments enhanced sE-CAD formation by 3.21 ± 0.44- and 3.43 ± 0.56-fold, respectively (Fig. 6C). Copper did not alter the levels of cell-associated full-length Jagged1 or E-cadherin protein (Fig. 6D) nor did the metal have any effect on the levels of RNA encoding these proteins (Fig. 6E).

Collectively, these results have shown that physiologically relevant and subcytotoxic copper concentrations promote the ectodomain shedding of endogenous Jagged1 and E-

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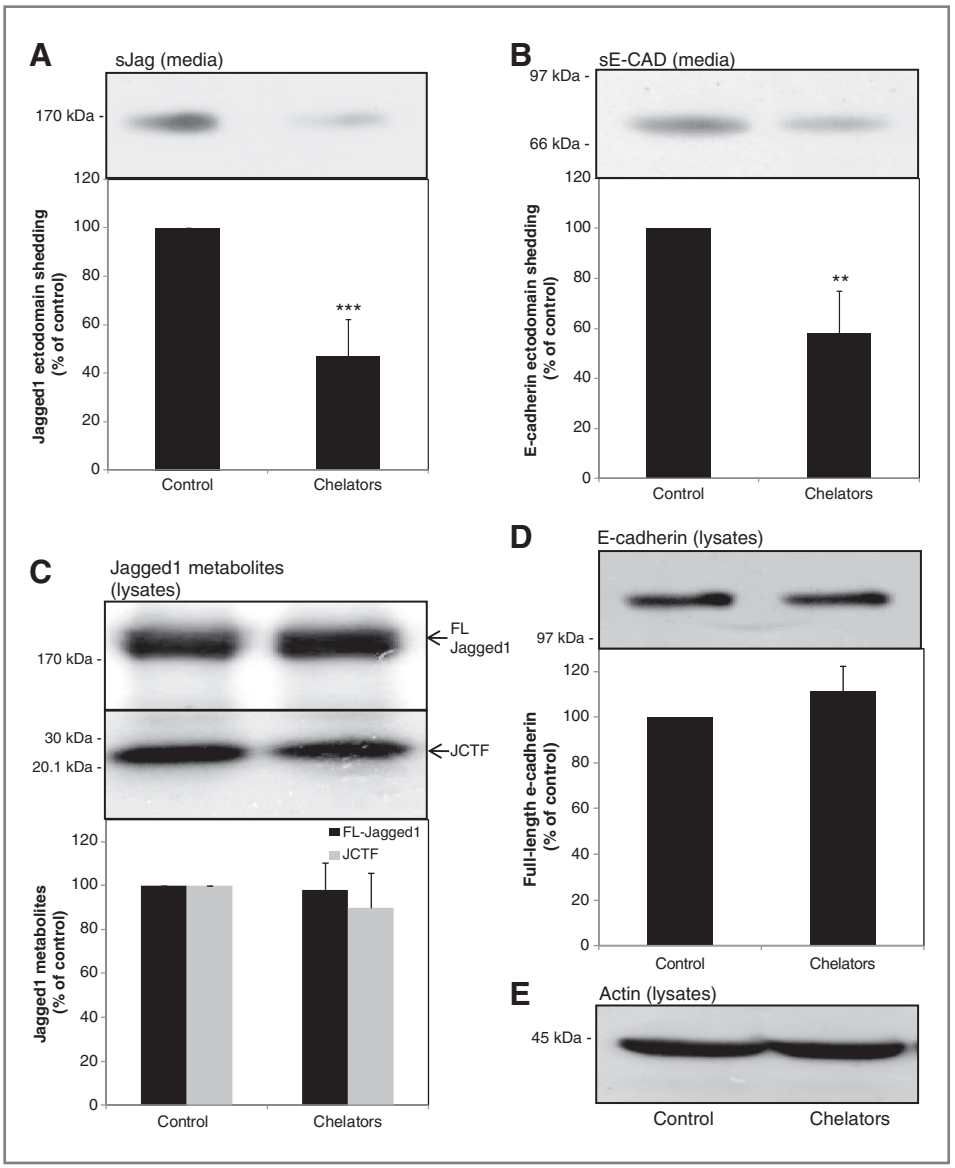

Figure 5. Chelation of endogenous copper impairs the shedding of Jagged1 and E-cadherin from HEK-Jagged1 cells. Cells were incubated for 5 hours in the presence or absence of 200 μmol/L final concentrations of bathocuproine and D-penicillamine before immunoblotting the subsequently prepared conditioned medium and lysates with antibodies against the indicated proteins. A and B, conditioned medium was immunoblotted with anti-Jagged1 ectodomain (A) and anti–E-cadherin ectodomain (B) antibodies, respectively. C–E, lysates were immunoblotted with anti-Jagged1 C-terminal (C), anti–E-cadherin ectodomain (D), and anti-actin (E) antibodies. Immunoblots are representative of 3 independent experiments and were quantified by densitometric analysis. Results are means ± SD ($n = 3$). **, $P \leq 0.01$; ***, $P \leq 0.005$; unless otherwise indicated, results were not significantly different.

cadherin from PC3 cells without enhancing overall protein/RNA expression levels.

Copper enhances the invasion of PC3 cells

Given the predilection of prostate cancer to metastasize to the BMS, we examined the effect of copper on the invasion of PC3-GFP cells in a model system designed to mimic the blood/BMS boundary (Fig. 7A and Materials and Methods; ref. 30). The results (Fig. 7B) clearly show that the invasion of PC3-GFP cells toward BMS was much greater than toward TCP alone. Furthermore, when copper was incorporated into the assays, significantly enhanced invasion toward BMS was observed after only 3 hours and persisted for the entire 24 hours time course.

We next examined the ability of ilomastat to impair the copper-stimulated invasion of PC3-GFP cells using final endpoint (24 hours) readings (Fig. 7C). As observed previously, invasion toward BMS was much greater than toward TCP alone. When the assays were conducted in the presence of BMS and ilomastat, no significant decrease in invasion was apparent. As expected, when copper was incorporated into the assay, invasion toward BMS was significantly enhanced and this increase was ablated by coincubation with copper and ilomastat.

Finally, having established that exogenous copper could promote PC3-GFP invasion, we examined whether the chelation of endogenous copper could invoke a reciprocal effect. The results (Fig. 7D) clearly show that the invasion of PC3-GFP cells toward BMS could be completely ablated through the inclusion of bathocuprione and D-penicillamine (200 μmol/L) in the assays. PC3-GFP viability was not affected by the presence of either ilomastat or the copper chelators used in the study (data not shown).

Published OnlineFirst on August 30, 2012; DOI:10.1158/1541-7786.MCR-12-0312

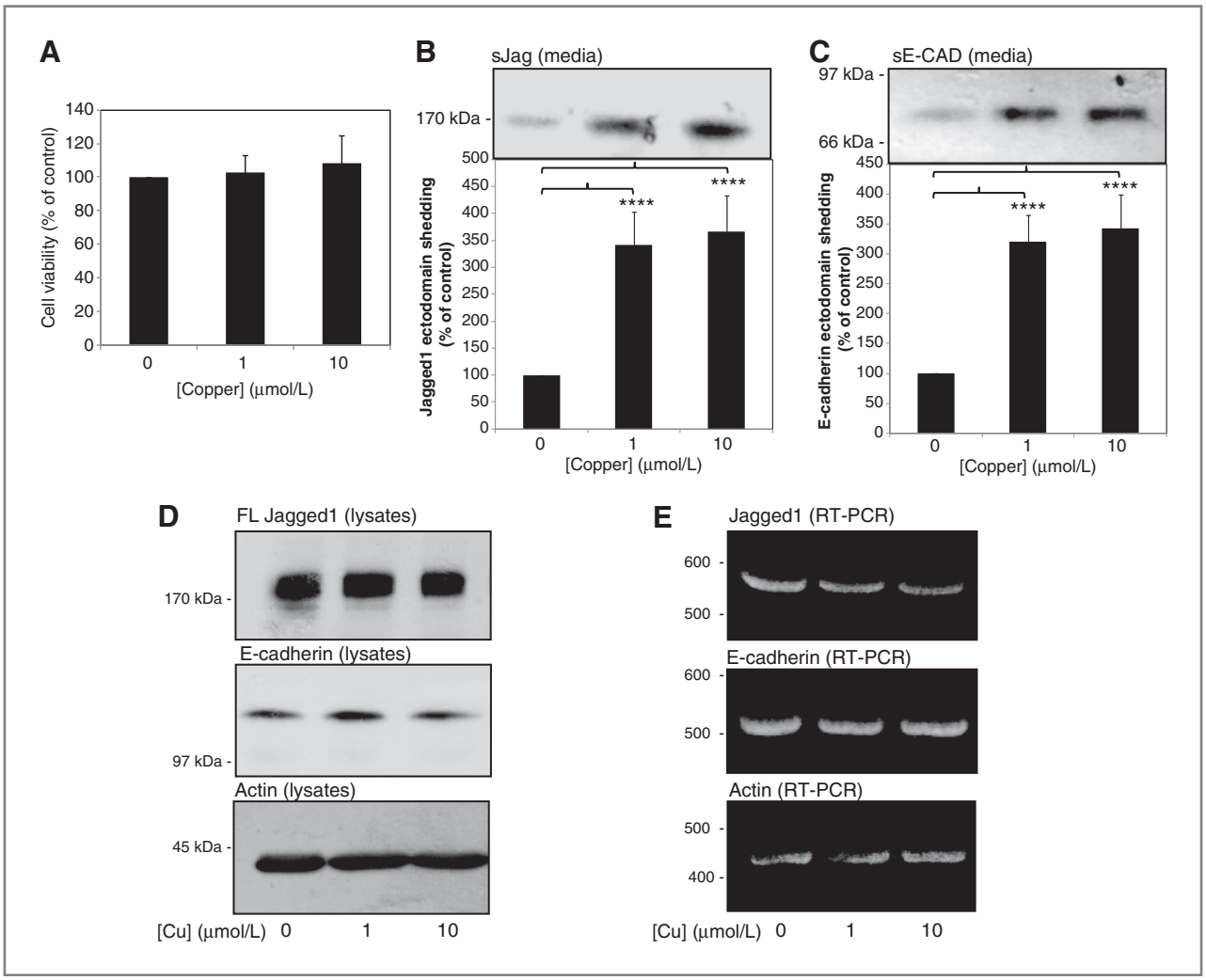

Figure 6. Copper promotes the zinc metalloproteinase-regulated ectodomain shedding of endogenous Jagged1 and E-cadherin in PC3 cells. Cells were incubated for 24 hours in the presence of the copper concentrations indicated. A, MTS assay showing a lack of cytotoxicity at either of the copper concentrations employed. B and C, conditioned medium was immunoblotted with anti-Jagged1 ectodomain (B) and anti–E-cadherin ectodomain (C) antibodies, respectively. D, cell lysates were immunoblotted with anti-Jagged1 C-terminal and anti–E-cadherin antibodies to detect the full-length forms of the proteins. Lysate samples were also immunoblotted with anti-actin antibody. E, RT-PCR of Jagged1, E-cadherin, and actin RNA. Immunoblots and RT-PCR data are representative of 3 independent experiments and the former was quantified by densitometric analysis. Results are means ± SD ($n = 3$). ****, $P \leq 0.001$; unless otherwise indicated, results were not significantly different.

Collectively, these results show that the effects of copper and copper chelators on protein shedding in PC3 cells are mirrored by the effects of these compounds on cell invasion.

Discussion

Despite its known proangiogenic role (34), little more is known about how, at the molecular level, copper might promote cancer progression. We have investigated whether copper could promote the proteolysis of key signaling and adhesion molecules previously linked to the progression of prostate cancer and whether, at the same concentrations, the metal could stimulate PEC invasion.

Jagged1 expression has been linked to the proliferation of various PECs and to the metastasis and recurrence of prostate cancer (15–17). In the current study, copper did not alter the levels of full-length cellular Jagged1, so it is unlikely that Notch activation would be altered as a consequence of altered full-length ligand availability. However, copper concentrations as low as 1 μmol/L enhanced the ZMP-mediated generation of sJag (Figs. 1 and 6). What the consequences of enhanced sJag levels might be for PECs is unclear; repression of transmembrane Jagged1 signaling by the soluble form has been suggested (35, 36) while soluble Jagged1 has been shown to promote *in vivo* tumorigenicity of fibroblasts (37). The absence of a reciprocal decrease in cell-associated Jagged1 might be explained by the fact that only a minor fraction of the protein is shed from the cell surface (10) and/or that a feedback loop exists to replenish expression of the protein (perhaps via the increased generation of JICD). At least one other RIP substrate, APP, can regulate its own

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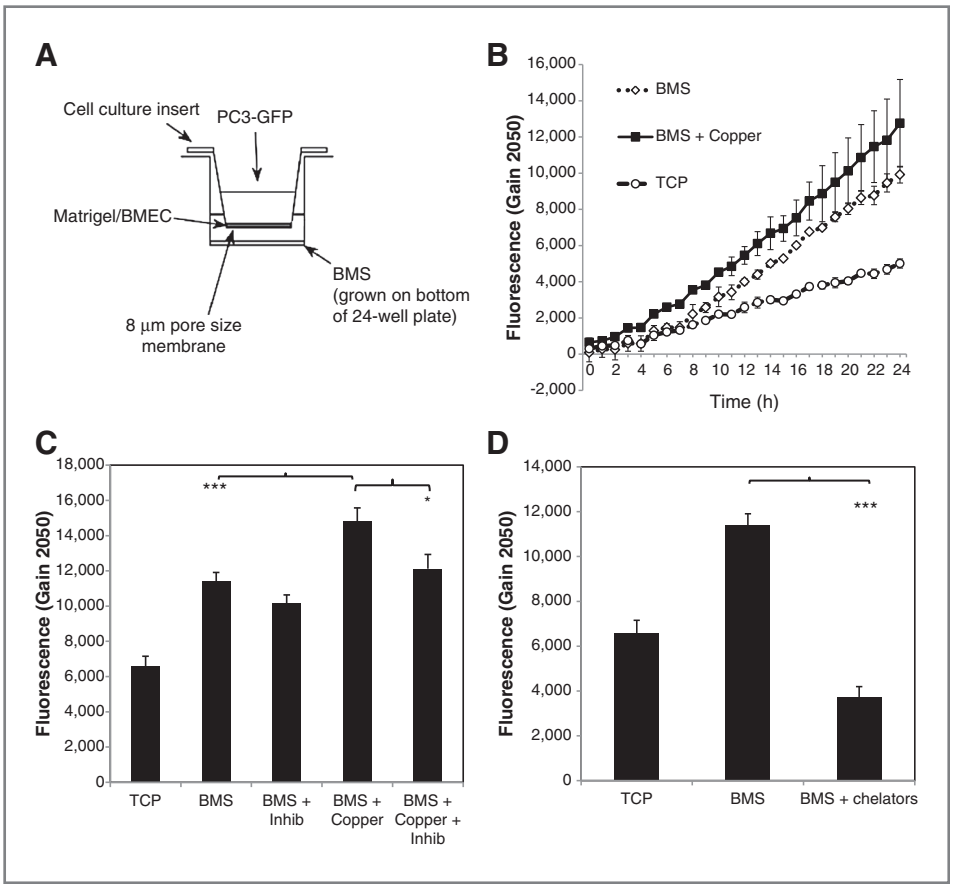

Figure 7. Copper enhances the invasion of PC3-GFP cells. Cells were preincubated for 5 hours with copper (10 μmol/L), ilomastat (5 μmol/L), or copper chelators (200 μmol/L) as indicated, before seeding the cells onto a BMEC/Matrigel support (A) and continuing to incubate in the absence or presence of the same reagents, whereas monitoring invasion toward TCP alone or BMS. B, the invasion of PC3-GFP cells was monitored in the absence/presence of copper over a 24 hours time course. C, endpoint (24 hours) monitoring of PC3-GFP cell invasion in the absence/presence of copper and/or ilomastat. D, endpoint (24 hours) monitoring of PC3-GFP cell invasion in the absence/presence of copper chelators. Results are means ± SEM ($n = 6$). *, $P \leq 0.05$; ***, $P \leq 0.005$; unless otherwise indicated, results were not significantly different.

expression via AICD (APP intracellular domain) signaling (38). It should also be noted that, at higher, physiologically irrelevant copper concentrations a reciprocal decrease in cell-associated Jagged1 holoprotein was observed (Supplementary Fig. S1).

As for the mechanism of sheddase activation, we have shown (Fig. 2C) that the treatment of HEK-Jagged1 cells with cycloheximide did not alter the ability of copper to stimulate Jagged1 shedding indicating that *de novo* synthesis of sheddases was not involved and that the metal enhanced the activity of preexistent ZMPs. Furthermore, we have shown that copper enhanced Jagged1 proteolysis in a cell-free membrane system (Fig. 2D). These latter data further support the argument that *de novo* sheddase synthesis is not required and also indicate that enhanced trafficking of either sheddases or sheddase substrates is not involved. Thus, it is clear that copper promotes shedding via a mechanism involving the posttranslational activation of "classic" ZMP sheddases. Interestingly, when SDS-PAGE gels were subjected to extended run times, a decrease in the size of the sheddase-cleaved Jagged1 ectodomain was apparent in the conditioned medium from copper-treated cells (Supplementary Fig. S2). This suggests either a change in the cleavage specificity of the constitutive Jagged1 sheddase, ADAM17 (10), or cleavage at a new site by an alternative ZMP. The fact that ADAM17 constitutively cleaves Jagged1 but not E-cadherin (10, 13) (both events being stimulated by copper) would argue against an altered cleavage site specificity of a single constitutive sheddase, as would the fact that the shedding of APP, which can be cleaved by ADAM17 (33), was not stimulated at copper concentrations of less than 200 μmol/L (Fig. 4). It, therefore, seems likely that the activity of one or more alternative ZMPs is enhanced in the presence of copper. In this respect, we have examined the effect of copper on Jagged1 shedding in HEK-Jagged1 cells transiently transfected with a number of candidate copper-stimulated members of the ADAM family (Supplementary Table S1). Cells transfected with the constitutive Jagged1 sheddase, ADAM17, did not exhibit enhanced copper-stimulated sJag generation nor did cells transfected with ADAM12-S or ADAM12-L, the former of which is activated via copper-mediated oxidation of an unpaired Cys273 in its catalytic domain (39). Of the remaining catalytically active human ADAMs, ADAM8, and ADAM19 also have an unpaired Cys residue in their catalytic domain; however, neither of these enzymes facilitated the effect of copper on sJag generation. Thus, the identity of the ZMP(s) which cleave Jagged1 and E-cadherin in the presence of copper remains unknown although the activities of several members of the MMP family are known to be enhanced by the metal (40, 41).

Under basal conditions, only a minor fraction of JCTF is processed to the JICD suggesting that the former fragment has a long half-life perhaps as a consequence of it being a poor

Published OnlineFirst on August 30, 2012; DOI:10.1158/1541-7786.MCR-12-0312

γ-secretase substrate or being spatially separated from the enzyme. Thus, the generation of a smaller ectodomain in the presence of copper might be expected to result in the accumulation of a slightly larger residual JCTF in the cell membrane; this was, however, not the case. One possible explanation is that, in the presence of copper, an alternative (larger) JCTF is generated which is a more effective γ-secretase substrate and, therefore, rapidly converted to the JICD without accumulating to a detectable steady-state level. Certainly, the fold increase in JICD generation at higher copper levels (30 μmol/L) did mirror that of the Jagged1 ectodomain (Figs. 2 and 3) thereby suggesting that all the JCTF generated in the presence of copper was being processed by γ-secretase. The fact that levels of JICD were enhanced by copper raises the possibility of broad ranging transcriptional changes in response to the metal that may affect a host of cellular attributes including invasive potential.

The fact that both Jagged1 and E-cadherin shedding were enhanced by copper but not manganese or iron indicates that redox activity was not mechanistically involved. Furthermore, the free radical scavenger DMSO and the antioxidants, *N*-acetyl-cysteine and ascorbate did not abrogate the effects of copper (data not shown). While ADAMs and MMPs rely on the active-site coordination of zinc for their catalytic activities, the metal did not enhance shedding. It is likely that, under the experimental conditions employed, the sheddase active sites were zinc-saturated such that enhancing the metal concentration further would not have stimulated enzyme activity. However, the coordination of zinc already *in situ* within the sheddase active sites could clearly abrogate copper-mediated shedding as evidenced by the ability of ilomastat to inhibit the phenomenon (the hydroxamic acid group of ilomastat forms a bidentate complex with active site zinc). The inclusion of calcium in our study was based primarily on the fact that the metal regulates activation of the Notch receptor, a process which might well impact on Jagged1 receptor binding and proteolysis (42); no calcium-mediated proteolysis of Jagged1 was, however, observed. The specificity of copper for promoting shedding is further exemplified by the fact that magnesium, a known cofactor for more than 300 enzymes, was also unable to activate the process.

As far as E-cadherin is concerned, copper did not alter cell-associated levels of the protein despite enhanced sE-CAD generation in the presence of the metal (Figs. 4 and 6). This observation might also be explained in terms of the arguments applied to Jagged1 earlier. However, the implications of enhanced soluble E-cadherin generation might be easier to predict than for sJag given the fact that the former protein is a cell adhesion molecule. In this respect, other studies have shown that sE-CAD can disrupt cell adhesion, cell aggregation, and support invasion, migration, proliferation, and survival (13).

Perhaps the key question arising from this study relates to the molecular mechanism(s) by which copper promotes the invasion of PECs. Our invasion assay results using the sheddase inhibitor, ilomastat, and copper chelators (Fig. 7) are particularly pertinent in this respect. In the absence of exogenous copper, ilomastat did not inhibit cell invasion (whereas it did so in the presence of the metal; Fig. 7C) indicating that ADAMs/MMPs require a threshold concentration of the metal to mediate their proinvasive effects. On the other hand, copper clearly regulates other molecular mechanisms contributing to cell invasion as copper chelators were able to completely ablate the stimulatory effect of BMS on PEC invasion in the absence of exogenous copper (Fig. 7D). Thus, it is apparent that enhanced shedding activity is one, but not the only, mechanism by which copper promotes the invasion of PECs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: E.T. Parkin
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.A. Parr-Sturgess, C.L. Tinker, C.A. Hart, E.T. Parkin
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.A. Parr-Sturgess, C.A. Hart, M.D. Brown, E.T. Parkin
Writing, review, and/or revision of the manuscript: C.A. Parr-Sturgess, M.D. Brown, N.W. Clarke, E.T. Parkin
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N.W. Clarke
Study supervision: E.T. Parkin

Grant Support

This research was supported by North West Cancer Research Fund (22 Oxford Street Liverpool L7 7BL, United Kingdom) grant code CR774.

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Received May 15, 2012; revised August 22, 2012; accepted August 23, 2012; published OnlineFirst August 30, 2012.

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