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Supplemental Data

The Serine Protease Matriptase 2 Inhibits Hepcidin Activation by Cleaving Membrane Hemojuvelin

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Supplemental Experimental Procedures

Immunofluorescence analysis

HeLa cells were grown for 16 hrs before transfection on glass cover slips in 12-well plates. Transfections were carried out using 1 µg (microgram) of plasmid DNA and 3 µl of the liposomal transfection reagent Lipofectamine in 1 ml of OptiMem, according to the manufacturer's instructions. After 12 hrs, the medium was replaced and, 24 hrs later, cells were washed with PBS and fixed in 4% paraformaldehyde/PBS for 30 min at room temperature, followed by three PBS washes. Cells were permeabilized by incubation in PBS containing 0.2% Triton X-100 and 10% donkey serum for 1 hr. The cells were then incubated for 2 hrs at room temperature with rabbit polyclonal anti-FLAG (1:250 dilution; Santa Cruz). After PBS washing, primary antibodies were visualized using the donkey anti-rabbit conjugated with Alexa Fluor 594 (Molecular Probes, Eugene, OR). Cover slips were mounted on glass slides using Dako Mounting Medium (Dako, Carpinteria, CA) and visualized at a 63X magnification under confocal microscope BioRad Leica TCS SP2 AOBS (Leica Microsystems, Bannockburn, IL).

Cloning strategies

Mutagenesis on matriptase-2 wild type was performed using the following oligonucleotides:

R774C sense: 5’ GGCACTCAGTGGCTGCTGGTTCCTGGC 3’

R774C antisense: 5’ GCCAGGAACCAGCAGCCACTGAGTGCC 3’

The matriptase-2^{MASK} variant, lacking the serine protease domain (Du et al, 2008), was obtained by amplification from the wild type cDNA by using the following primers:

MASK-sense: 5’ GGAATTCTGAGCTGTCTCGGC 3’ (EcoRI tail)

MASK-antisense:

5’GCTCTAGATCACTTGTCATCGTCGTCCTTGTAGTCAAGCTTGCCACAGTC ACAGTGC 3’ (FLAG, XbaI tail).

The amplicon was cloned into the EcoRI and XbaI sites of pcDNA3.1(+).

Expressing vector encoding uromodulin cDNA (UMOD, cMYC tagged) was a gift of Dr. Luca Rampoldi (Dulbecco Telethon Institute, Molecular Genetics of Renal Disorders, San Raffaele Scientific Institute, Milan, Italy).

Incubation of s-HJV with matriptase-2-expressing cells

HeLa cells were transiently transfected for 18 hrs with HJV^{wt} expressing vector as descrived above, and then incubated in serum-free media. After 24 hrs, media were collected, centrifugated for 10 min at 800 g to eliminate detached cells and filtered at 0.22 μm.

Matriptase-2^{wt} ($MT2^{wt}$) or mock- transfected cells were then incubated with the HJV-derived culture media. After 24 hrs, media were collected, concentrated using 5 kDa molecular weight (MW) cutoff ultrafiltration (Amicon Ultra; Millipore, Billerica, MA), and analyzed by SDS-PAGE.

Incubation of s-matriptase-2 with HJV-expressing cells

HeLa cells were transiently transfected for 18 hrs with matriptase-2^{wt} ($MT2^{wt}$) or empty vectors, and then incubated in serum-free media. After 24 hrs, media were collected, centrifugated for 10 min at 800 g to eliminate detached cells and filtered at 0.22 μm.

HJV^{wt} transfected cells were then incubated with the MT2- or mock-derived culture media. After 24 hrs, media were collected and concentrated using 5 kDa molecular weight (MW) cutoff ultrafiltration (Amicon Ultra; Millipore, Billerica, MA). Cells were incubated with PI-PLC as described above for m-HJV analysis, and then lyzed. Whole cellular extracts, PI-PLC-derived samples and media were then analyzed by SDS-PAGE.

Legend to Figures

Figure S1. Immunofluorescence analysis of wild type and mutants matriptase-2. HeLa cells were transiently transfected and analysed for immunofluorescence. Matriptase-2 (MT2) was revealed by anti-FLAG antibody. UP: unpermeabilized cells; P: Triton X-100 permeabilized cells. The white Bar represents 10 μm.

Figure S2. HeLa cells were cotransfected with the GPI-anchored protein uromodulin cDNA and matriptase-2 expressing plasmid, or the empty vector. Whole cellular extracts, concentrated media and PI-PLC-derived supernatants were analyzed by Western blot. Anti-cMYC was used to detect uromodulin. s-UMOD indicates soluble and m-UMOD membrane-associated uromodulin. The equal loading was verified by α-tubulin.

Figure S1

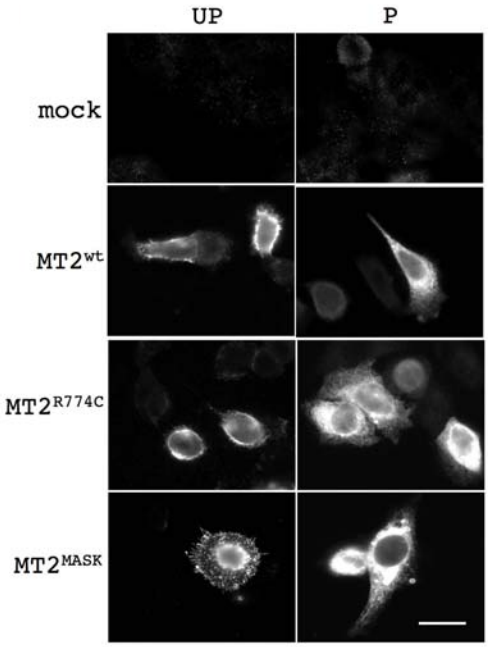
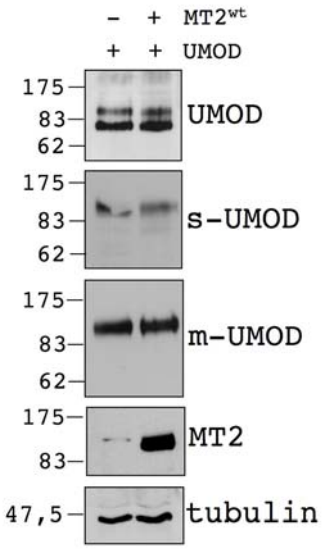


Figure S2


- + MT2wt
+ + UMOD
175
83
62
UMOD
175
83
62
s-UMOD
175
83
62
m-UMOD
175
83
MT2
47,5
tubulin