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Supplemental Data

Mutation of Membrane Type-1 Metalloproteinase, *MT1-MMP*, Causes the Multicentric Osteolysis and Arthritis Disease Winchester Syndrome

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Supplemental Material and Methods

Reagents & Antibodies

Rabbit polyclonal antibody (AB6004) against the hinge region of MMP14 was from Millipore (Billerica, MA). The rabbit polyclonal antibody (sc-25778) against the full length GAPDH was from Santa Cruz (Santa Cruz, CA). The rabbit polyclonal antibody (ab6529) against the c-terminal amino acids of pan cadherin was from Abcam (Cambridge, MA). The mouse monoclonal antibody (R96025) against a V5 epitope was from Invitrogen (Grand Island, NY). Cycloheximide was obtained from Sigma (St. Louis, MO). MG-132 was obtained from Calbiochem (Gibbstown, NJ).

Cell lines

Winchester (GM02295), GM17071 and AG06062 primary fibroblast cells were obtained from the Coriell Institute. All of these cells were grown in Eagle's minimum essential medium with Earle's salts and L-glutamine (Cellgro, Manassas, VA) with 15% fetal bovine serum (Gemini Bioproducts, West Sacramento, CA) and penicillin/streptomycin (Cellgro). COS-7 cells were obtain from ATCC and grown in Dulbecco's modified Eagle's medium (Cellgro) with 10% fetal bovine serum and penicillin/streptomycin. All cells were grown under standard conditions, 37^{o}C and 5% CO_2.

Gelatin Zymography

Cells were grown in serum-free medium and conditioned medium was collected at either 24 h or 48 h. The conditioned medium was concentrated by centrifugation in an Amicon Ultra-15 filter (Millipore). Equal amounts of concentrated conditioned medium and run on a 10% gelatin zymogram gel (Invitrogen), developed overnight and stained with Coommassie blue according to manufacter's instructions.

Primers

Sequencing primers:	
MMP14 ex1F	5'-GGTCTAGGAATTCAAGTTCAGTG-3'
MMP14 ex1R	5'-CAGTTGTAAAGTTCTTCCAAGG-3'
Cloning primers	
MMP14 mRNA1F	5'-GAGCCCACACTGCCCGGCTGACC-3'
MMP14 mRNA10R	5'-GGACAAGGTCTGACGCCCACCGCCG-3'
Real-time primers:	
MMP14 RT1F	5'-AGGAGACACCCACTTTGACT-3'
MMP14 RT1R	5'-GCCTTCTGTTCCTGATAAACCC-3'
MMP14 RT2F	5'-AGGAGAGAGCGAGAGAGG-3'
MMP14 RT2R	5'-CCCGAAGTAAGTGAGCTTC-3'

DNA extraction, PCR, and Sanger sequencing

Genomic DNA was extracted from patient-derived fibroblast cell lines (Qiagen, Valencia, CA) and the 13 exons, intron/exon boundaries, and flanking 5' promoter and 3' untranslated regions of the *MMP2* gene directly sequenced in both directions using the ABI BigDye terminator sequencing kit (Perkin-Elmer, Waltham, MA) as we have previously described. The 10 exons, intron/exon boundaries, and flanking 5' promoter and 3' untranslated regions of the *MMP14* gene were subsequently sequenced as well. Resulting sequence traces were analyzed using the program Sequencher 4.1 (GeneCodes).

DHPLC screening

Genomic DNA was isolated from 98 unrelated control subjects (50 of Puerto Rican descent, 48 of Caucasian descent) and screened for the presence of the identified mutation, using denaturing high-performance liquid chromatography (DHPLC) (Transgenomic, Omaha, NE). All samples with evidence of heteroduplex formation were then directly sequenced as described above.

Immunoblot Analysis

Cell extracts were harvested in radioimmunoprecipitation assay buffer (Santa Cruz Biotechnology; standard protocol). Equal amounts of protein (determined by the Bio-Rad DC protein quantification assay) were loaded and separated by PAGE and transferred to nitrocellulose membranes and probed with the respective antibody. Enhanced chemiluminescent immunoblot images are shown with amounts normalized to control proteins and then expressed as relative intensities determined by Image J software and reported in the figure legends. Each experiment was run three separate times and technical replicates performed and representative immunoblots are shown.

Transient Transfections

Vector backbones (pcDNA3.1/V5-His TOPO TA-Invitrogen, #K4800) were purchased and full-length WT MMP14 cDNA and Winchester MMP14 were ligated in according to manufacturer's protocol. COS-7 cells were plated at 3 $\times 10^5$ cells/well in 6-well plates. Transfections of DNA with the X-tremeGENE 9 (Roche, Indianapolis, IN) were performed per manufacturer's instructions in duplicate wells 24 h post-plating. Briefly, approximately 4 μg of DNA and 12 μl X-tremeGENE 9 were diluted in serum-free DMEM and then combined. This mixture was added to cells with DMEM containing serum. Cells were collected at either 24 or 48 h following transfection.

Cellular Fractionation

Cells were collected from five 10 cm dishes per cell type in homogenization buffer (10 mM Tris HCl pH 7.4, 1 mM ETDA, 0.5% Triton X-100, 1 mM PMSF and protease inhibitor), lysed and incubated on ice. Cell debris was removed by centrifugation at 900 x g. The remaining supernatant was centrifuged at 30,000 RPM for 75 min. The cytoplasmic fraction (supernatant) was removed and the membrane fraction (pellet) was re-suspended in buffer (10 mM Tris HCl pH 7.4, 1 mM ETDA, 0.5% Triton X-100, 1 mM PMSF and protease inhibitor). Insoluble material was removed by centrifugation and the membrane fraction was collected.

Cell Surface Protein Isolation

Cell surface biotinylation was performed with primary cells using the Pierce Cell Surface Protein Isolation kit (Thermo Scientific, Rockford, IL) according to the manufacturer's protocol. Briefly, cells were grown to approximately 90% confluence on four 10-cm culture dishes. These dishes were washed with cold PBS and incubated with EZ-LINK Sulfo-NHS-SS-biotin for 30 min at 4 °C. Next a quenching solution was added then cells were lysed with lysis buffer containing protease inhibitors. The biotinylated proteins were isolated with NeutrAvidin agarose gel. Sample buffer with DTT was used for elution.

Real-time PCR

Total RNA was extracted using RNeasy mini kit (Qiagen) and treated with DNase (Qiagen) according to the manufacturer's instructions. cDNA synthesis was carried out with 1 μg of total RNA per sample using random primers and the Iscript kit (Bio-Rad, Hercules, CA). Quantitative PCR was perfomed on an ABI Prism 7900HT sequence detection system (Applied Biosystems, Carlsbad, CA). Cycle number values were normalized with GAPDH, β-actin and 18S values. Values were analyzed and fold-change compared with empty vector or WT MMP14 vector control values. Data shown are the average of three separate experiments, each done in triplicate.

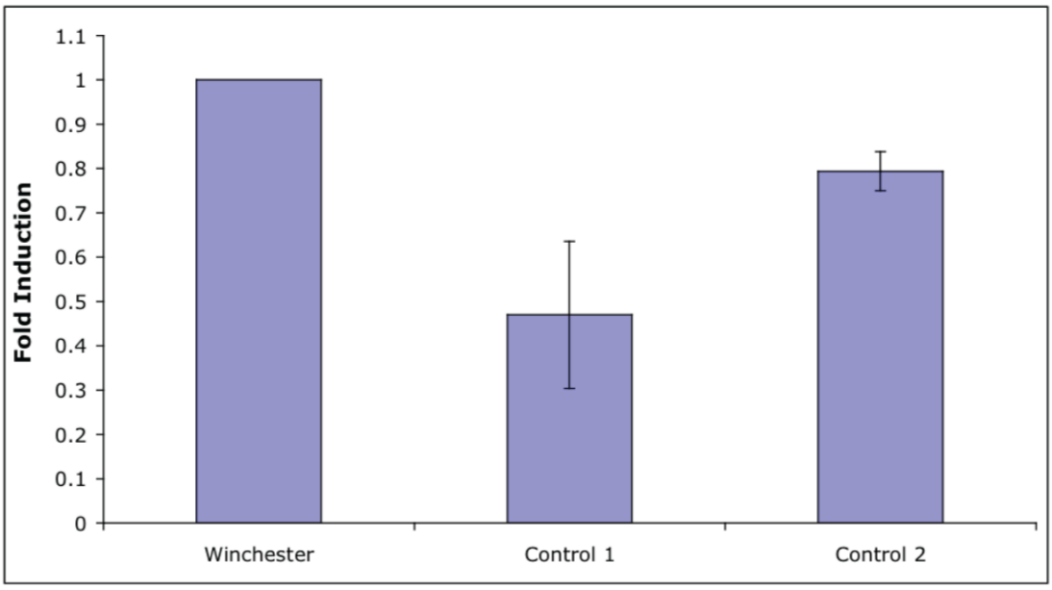

Figure S1. MT1-MMP mRNA Levels Are Comparable between Winchester Primary Fibroblasts and Control Primary Fibroblasts

Real-time PCR confirms that the reduction in MT1-MMP protein levels in Winchester cells is not due to a relative decrease in MT1-MMP mRNA compared to control levels. Error bars represent the standard deviation of triplicate experiments.

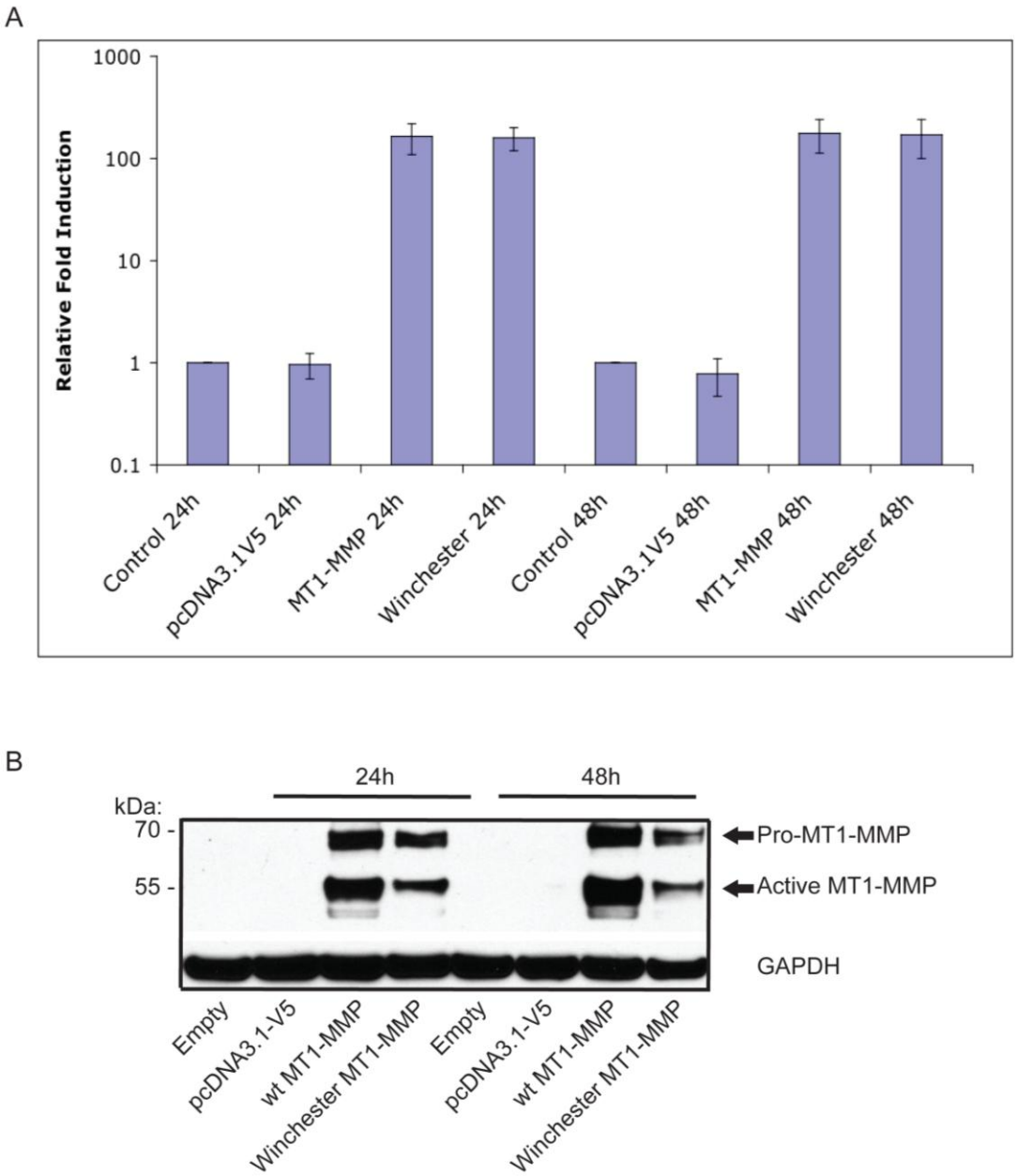

Figure S2. Transient Transfections with WT MT1-MMP or Winchester MT1-MMP Expression Constructs Were Performed in COS-7 Cells

(A) Real-time PCR demonstrates >100 fold greater expression of MT1-MMP in cells transfected with either WT or mutant MT1-MMP. Error bars represent the standard deviation of triplicate experiments.

(B) Immunoblot reveals a greater expression of pro-enzyme and the active form in cells transfected with WT MT1-MMP compared to mutant MT1-MMP.

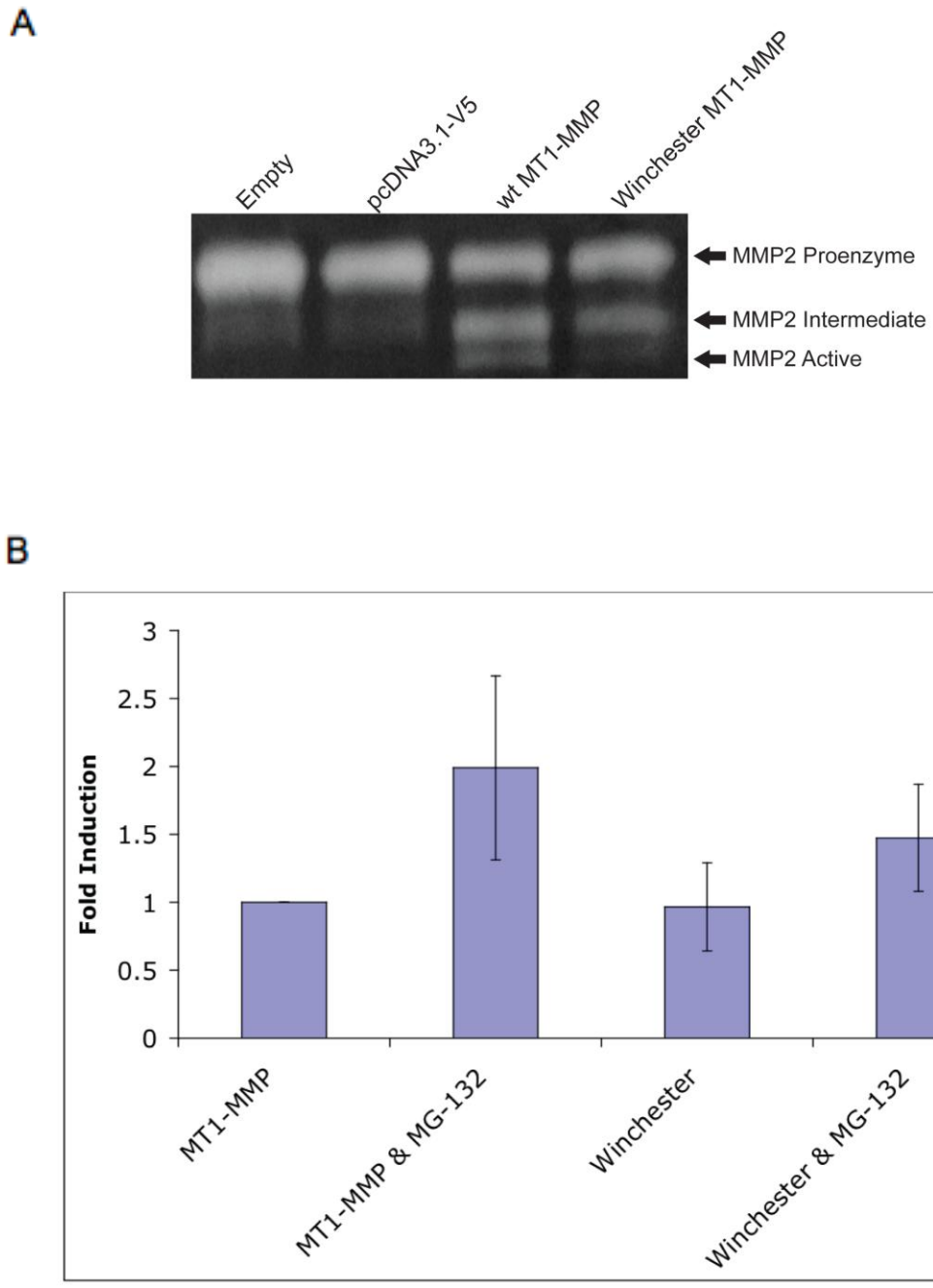

Figure S3. Zymogram and Real-Time PCR Analysis of Transiently Transfected COS-7 Cells

(A) Zymogram with conditioned medium from WT MT1-MMP has more active MMP2 than medium from altered MT1-MMP.

(B) Real-time PCR shows MG-132 had no effect on the expression of *MT1-MMP* mRNA. Error bars represent the standard deviation of triplicate experiments.