# Cathepsin X is secreted by human osteoblasts, digests CXCL-12 and impairs adhesion of hematopoietic stem and progenitor cells to osteoblasts

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## Online Supplementary Design and Methods

### Antibodies, proteases and recombinant proteins

Recombinant human cathepsin X and cathepsin B were purchased from R&D Systems (Wiesbaden, Germany). The CXCL-12 isoforms SDF-1α (68 amino acids) and SDF-1β (72 amino acids) were obtained from Peprotech (Hamburg, Germany). Polyclonal rabbit antisera targeting either the prodomain or the catalytic domain of human cathepsin X were purchased from Abcam (Cambridge, UK), whereas the monoclonal antibody targeting the catalytic domain of cathepsin X was obtained from R&D Systems.

### Human primary cells and cell lines

Hematopoietic stem and progenitor cells (HSPC) were isolated from umbilical cord blood samples in accordance with the guidelines approved by the local ethics committee. Cord blood mononuclear cells were purified using Percoll (1.077 g/mL) density gradient centrifugation and labeled with CD34-binding MACS microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. For magnetic enrichment the automated magnetic activated cell sorting unit AutoMACS (Miltenyi) was used in the sensitive double-positive separation mode (PosselD2). The $CD34^+$ cell population obtained was highly enriched for HSPC. These freshly isolated $CD34^+$ HSPC could be either used immediately in the different assays or stored overnight in StemSpan® SFEM - serum-free expansion medium supplemented with StemSpan® CC100 - cytokine cocktail (StemCell Technologies, Cologne, Germany).

The human osteoblastic cell lines CAL72 (obtained from DSMZ, Braunschweig, Germany), MG63 (obtained from ATCC, Manassas, USA) and G292 (obtained from ECACC, Salisbury, UK) were grown in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS). Culture medium for CAL72 cells additionally contained an insulin-transferrin-selenium supplement (Gibco® Invitrogen).

Primary osteoblasts were isolated from bone waste of hip joint femoral heads as recently described.[1] Briefly, bone was mechanically minced and incubated in dispase II (Roche, Mannheim, Germany) and collagenase type XI (Sigma, Taufkirchen, Germany). After 1 h of incubation, the supernatant was discarded. The bone fragments were again incubated with collagenase for 3 h and, after washing, were transferred into cell culture flasks. The adherent primary osteoblasts were cultured in DMEM containing glucose supplemented with FCS, MEM vitamin solution, Fungizone (Invitrogen), β-glycerophosphate and ascorbic acid (Sigma) according to an established protocol[2] and routinely tested for osteogenic marker genes such as osteopontin (OPN), osteocalcin, RunX2, bone sialoprotein, collagen type I alpha2, osteonectin and alkaline phosphatase (ALP) using quantitative RT-PCR. Protein expression of OPN, ALP and RunX2 was confirmed by immunofluorescence staining. The primary cells were used in between passage one to five with the maximum of five passages. All studies were approved by the local ethics committee.

The established human bone marrow stromal cell lines L87/4 and L88/5[3] were cultured in RPMI 1640/10% FCS supplemented with hydrocortisone, whereas HS-5 cells[4] were cultured in RPMI 1640/20% FCS without hydrocortisone.

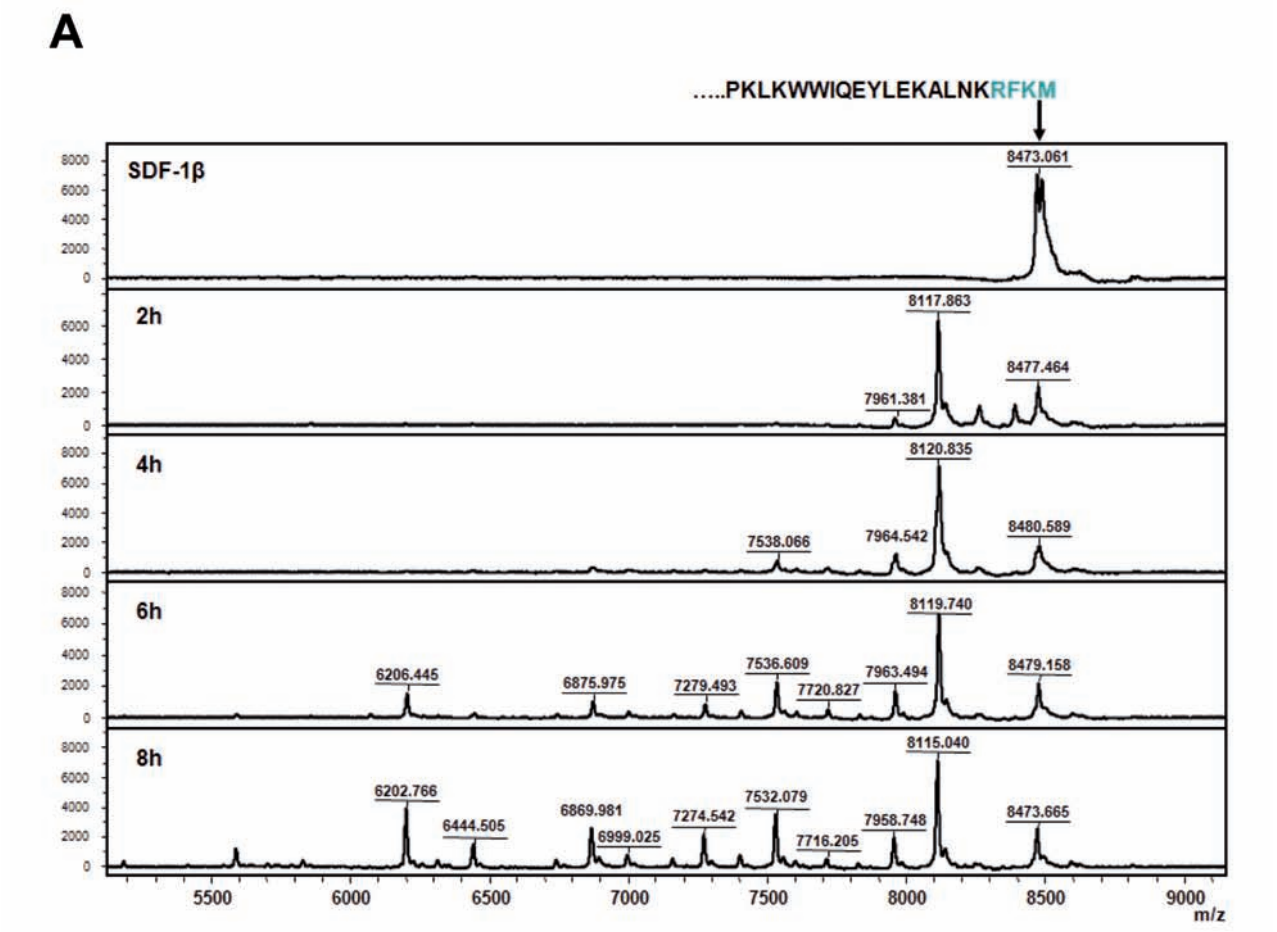
### Collection of conditioned media

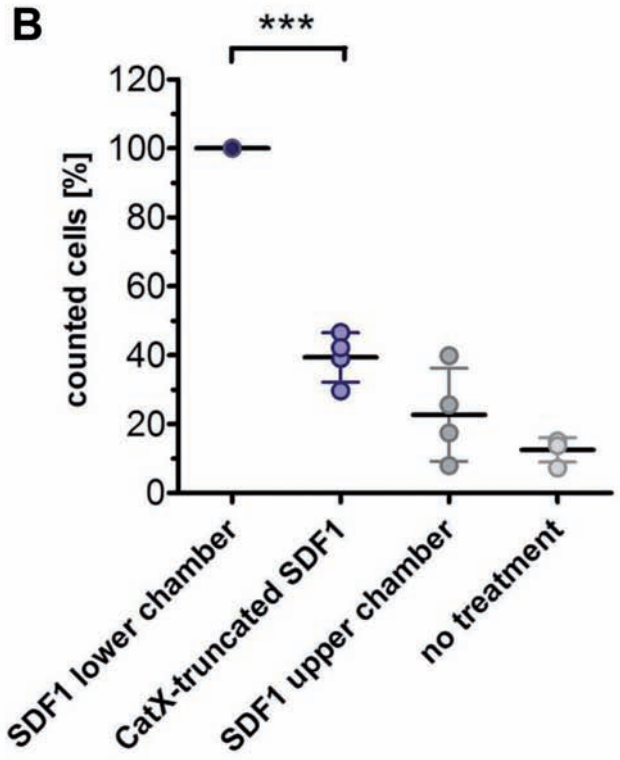
For the collection of osteoblast-conditioned media, $3.5\times10^6$ cells were allowed to adhere to tissue flasks overnight. On the following day the cells were gently washed with phosphate-buffered saline (Gibco) and then incubated in serum-free DMEM for 24 h. The conditioned media were harvested and concentrated ten-fold by ultrafiltration (cut-off 10 kDa) according to the recommendations of the manufacturer (Millipore Amicon, Schwalbach, Germany). Shock-frozen aliquots of the concentrated conditioned media were prepared to avoid subjecting the samples to repeated cycles of freezing and thawing.

### Reverse transcriptase-polymerase chain reaction analysis

Total RNA was isolated from cell pellets using the RNeasy®

total RNA kit (Qiagen, Hilden, Germany). Two micrograms of RNA of each sample were transcribed into cDNA using the SuperScript™ III First-Strand Synthesis System (Invitrogen) followed by a PCR using Taq DNA Polymerase (Roche Diagnostics). The following exon-overlapping primer pair for human cathepsin X (GenBank database accession number: NM_001336.3) was used: CatX-forward 5'-AAG GGG GTA ATG ACC TGT CC-3', and CatX-reverse 5'-CCT CGA TGG CAA GGT TGT AT-3'. After an initial denaturation step at 72°C for 120 sec, the PCR was run for 30 cycles (denaturation




**Online Supplementary Figure S1.** Cathepsin X cleaves SDF-1β and diminishes chemotaxis of CD34⁺ HSPC towards CXCL-12. (A) 3 μM of the CXCL-12 isoform SDF-1β was incubated at 37°C with 0.35 μM pre-activated cathepsin X for up to 8 h. Degradation of SDF-1 was stopped at different time points (2 h, 4 h, 6 h and 8 h) by adding 1% TFA. MALDI-TOF analysis showed that, although cathepsin X also digested this isoform, trace amounts of SDF-1 could still be found after 8 h. (B) CD34⁺ HSPC were allowed to migrate through laminin-511-coated filters towards media alone (*no treatment*), towards SDF-1 (*SDF1 lower chamber*) or towards SDF-1 pre-treated with activated cathepsin X (*CatX-truncated SDF1*). As an additional control SDF-1 was added only to the upper chamber (*SDF1 upper chamber*). The percentage of CD34⁺ HSPC migrating towards intact SDF-1α was set to 100%. The chemotactic effect of SDF-1 in the lower chamber was drastically reduced by digestion with the activated protease cathepsin X. The mean average of four independent experiments is shown.

at 94°C, 30 sec; primer annealing at 56°C, 40 sec; primer extension at 72°C, 60 sec). The amplified PCR products were visualized after 2% agarose gel electrophoresis and staining with ethidium bromide through UV light exposure.

### *Targeted knock-down of cathepsin X in primary osteoblasts*

The anti-human cathepsin X (NM_001336.3)-targeting stealth small inhibitory RNA (siRNA) CTSZ/HSS175722 (low GC), CTSZ/HSS102509 (med GC) and the scrambled control oligonucleotides were purchased from Invitrogen.

Eighty percent confluent osteoblast cell layers were transfected once in 48-well plates, with either 16 nM of the cathepsin X-targeting siRNA or the scrambled control siRNA using Lipofectamine RNAimax (Invitrogen) according to the manufacturer's advice. The knock-down efficiencies were checked in the cell lysates and the cell supernatants 2 or 3 days after transfection by western blotting.

### *Immunofluorescence staining*

Primary osteoblasts or osteosarcoma cells were seeded in eight-well chamber slides (BD Biocoat, Heidelberg, Germany), cultured to 80% confluence and fixed in 4% paraformaldehyde at 4°C. For intracellular staining of cathepsin X the cells were permeabilized with 0.1% Triton X-100. Staining was performed with either rabbit-anti-human cathepsin X pro-domain antiserum (Abcam) or monoclonal antibody directed against the catalytic domain of cathepsin X. After washing, cells were incubated with either secondary Cy2-labeled anti-rabbit antibodies or Cy3-labeled anti-mouse antibodies (Jackson ImmunoResearch Laboratories, Hamburg, Germany). Cell nuclei were counterstained with 4',6-diamino-2-phenylindol-dihydrochloride (DAPI). Control staining was performed by omitting the primary antibodies. The labeled cells were examined using an AxioObserver Z1 fluorescence microscope equipped with an ApoTome for optical sectioning (Zeiss, Göttingen, Germany). For double immunofluorescence, digital pictures were taken from each fluorescence channel and superimposed using the Axiovision™ software from Zeiss to detect specific antibody staining.

### *Immunoblotting*

Equal volumes of ten-fold concentrated conditioned cell culture media were loaded on 12% sodium dodecylsulfate-polyacrylamide gels and run under reducing conditions. After electrophoresis proteins were blotted onto polyvinylidene fluoride membranes. After blocking in 3% bovine serum albumin/0.1 % Tween 20 in Tris-buffered saline, the polyvinylidene fluoride membranes were probed overnight with the polyclonal anti-human cathepsin X antiserum (Abcam), followed by incubation with a goat anti-rabbit horseradish peroxidase-conjugated antibody (Abcam). Chemiluminescent detection of the bound antibodies was performed using the Millipore ECL detection system.

### In vitro *biotinylation of cell surface proteins*

One day prior to biotinylation, $5\times10^5$ cells/well were seeded on 6-well plates. Biotinylation was performed using 0.02 mg/mL N-hydroxysulfo-succinimide biotin ester (Calbiochem, Bad Soden, Germany). The cells were incubated with the biotinylation reagent for 10 min, washed with phosphate-buffered saline and subsequently quenched by incubation with 50 mM glycine. Cell lysis was performed with 500 µL lysis buffer (pH 5.5). Biotinylated cell surface proteins were precipitated overnight at 4°C using streptavidin beads. On the following day the beads were washed with 25 mM citrate solution (pH 5.0) and incubated in Laemmli buffer. After electrophoresis and transfer to polyvinylidene fluoride membranes, biotinylated cell surface-associated cathepsin X was visualized by immunoblotting with the anti-cathepsin X antiserum.

### *Cell-cell adhesion assay*

Primary osteoblasts were seeded on 48-well plates 1 day before the cell adhesion assay was performed. $CD34^+$-MACS-sorted HSPC were labeled with the fluorescent dye BCECF-AM (2′, 7′-bis (2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester, Sigma-Aldrich). After washing, $CD34^+$ HSPC were incubated on the adherent osteoblast cell layer for 40 min to allow adhesive interactions. During the 40 min incubation period, pro-cathepsin X (370 ng/20 µL) was separately activated in 25 mM sodium acetate (pH 3.5) containing 5 mM dithiothreitol.

After the initial attachment of $CD34^+$ HSPC to osteoblasts, either 92.5 ng of pre-activated or immature cathepsin X or buffer alone without dithiothreitol was added to the cells for 20 min. Then the cells were washed twice with phosphate-buffered saline to remove the non-bound $CD34^+$ HSPC. Adhesion of the remaining $CD34^+$ HSPC was measured with Fluoroskan Ascent (Thermo Scientific, Dreieich, Germany) at 405 nm and calculated as follows:

$$\frac{\text{fluorescence from experimental sample - fuorescence from negative control sample}}{\text{fluorescence of the positive control sample}} x100 = \%\ \text{cell adhesion}$$

To inhibit the cathepsin X-mediated effect on $CD34^+$ HSPC-osteoblast interactions, cathepsin X was incubated with the active-site labeling reagent DCG-04. Cathepsin X (370 ng) was activated for 5 min and the protease was pre-incubated for 40 min with either 0.66 mM DCG-04 (in dimethylsulfoxide) or dimethylsulfoxide alone as a control. All adhesion assays were carried out in triplicate.

### *Cell migration assay*

Polycarbonate membranes of a 96-well ChemoTx® plate with a pore size of 3 µm (Neuroprobe, Gaithersburg, MD, USA) were coated with 100 ng/µL laminin-511/521 (Sigma). The coated membranes were gently washed with phosphate-buffered saline before $1.7\times10^4$ umbilical cord blood-derived $CD34^+$ HSPC were added on top of the membranes. The lower chambers contained either no stimulus, SDF-1α at a concentration of 100 ng/mL or cathepsin X-digested SDF-1α (100 ng/mL). As an additional control, SDF-1α was applied to the upper chamber of the plate. For digestion of SDF-1α, 3 µM SDF-1α were incubated overnight with 50 nM cathepsin X. After 15 h of migration, the number of cells present in each lower chamber was determined by counting. Each experiment was performed in triplicate.

### *Statistical analysis*

Data are presented as means ± standard error of the mean

(SEM) or means ± standard deviation (SD). The significance was evaluated using a two-tailed Student's t-test considering $CD34^+$ HSPC-osteoblast-adhesion experiments (comparison between the control and mCatX or between mCatX and iCatX). Differences with *P* values less than 0.05 or less than or equal to 0.005 were considered to be statistically significant or highly significant (indicated with * or ***, respectively).

For the chemotaxis experiments we used column statistics, because the migration of $CD34^+$ HSPC towards the chemoattractive chemokine SDF-1 was set to 100% in each experiment. Differences with *P* values less than or equal to 0.0005 were considered to be highly statistically significant (indicated with ***).

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