Short Communication

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IGF-I receptor phosphorylation is impaired in cathepsin X-deficient prostate cancer cells

Abstract: The cysteine-type peptidase cathepsin X is highly upregulated in several cancers and presumably promotes tumor invasion through bypassing cellular senescence. Here, we present first evidence that the underlying mechanism may involve the regulation of the insulin-like growth factor (IGF) system, a well-known activator of proliferating tumor cells. Cathepsin X deficiency leads to a reduced phosphorylation of the IGF-I receptor in response to IGF-I stimulation. In addition, downstream signaling through focal adhesion kinase was also affected. Taken together, our results indicate that cathepsin X is able to assist in IGF signaling, which may be an important progress toward understanding cathepsin X-dependent tumorigenesis.

Keywords: growth factor; LNCaP; PC-3; protease; RNA interference; senescence.

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The insulin-like growth factor (IGF) pathway fulfills important functions in growth, development, and survival and has also been implicated in the pathogenesis of malignancies (Hanahan and Weinberg, 2000; Pollak, 2008). On the one hand, IGFs may promote excessive and uncontrolled cell division through the activation of cell cycle progression and inhibition of apoptosis, thereby contributing to cancer development. On the other hand, the modulation of the IGF axis can alter aging phenotypes and longevity in animal models as well as the life span of cells in culture (Kamrava et al., 2011).

Within the last decade, cellular senescence has emerged as a safety mechanism that irreversibly arrests the proliferation of damaged cells that are at risk for malignant transformation (Campisi, 2001; Smith and Kipling, 2004). Hence, cancer cells have adopted several ways to overcome this powerful tumor suppressor pathway. Besides mutations in senescence-relevant genes such as *p53*, one such mechanism seems to act through lysosomal cathepsins such as cathepsin X, which is upregulated in several cancers (Nägler et al., 2004; Krueger et al., 2005) and has been shown to participate in invasive processes (Sevenich et al., 2010). Remarkably, the downregulation of cathepsin X leads to accelerated cellular senescence (Kraus et al., 2011). Apart from bypassing cellular senescence, tumor invasion due to upregulated cathepsin X has also been suggested to be promoted through alternative mechanisms such as compensation for the loss of cathepsin B in primary cells (Vasiljeva et al., 2006) or the induction of epithelial-mesenchymal transition (Wang et al., 2011).

Evidence is accumulating that cathepsins play significant roles in signal transduction pathways (see, for example, Malla et al., 2010; Chen et al., 2012). Members of this group of enzymes carry mannose-6-phosphate (M6P) recognition moieties to be sorted to lysosomes *via* the cation-dependent M6P receptor (MPR). Cathepsins are also secreted by many cancers and can be reinternalized by interacting with the IGF-II/cation-independent MPR and thereby possibly modulating IGF signaling. Notably, the expression of IGFs inversely correlates with proliferative aging. For example, IGF-II levels are decreased in senescent human fibroblasts (Kanzaki et al., 2002), whereas IGF-I was shown to reduce the senescence of intervertebral annulus cells *in vitro* (Gruber et al., 2008), and its synthesis decreases in murine submandibular glands with aging (Kobayashi et al., 2004). Therefore, we hypothesized that alterations within the IGF axis could lead to the observed senescent phenotypes in cathepsin X-deficient cells. To test this hypothesis, we used RNA

interference to achieve selective knockdown of cathepsin X in two human prostate carcinoma cell lines (PC-3 and LNCaP) and analyzed its contribution to IGF receptor activation. Here we present first evidence implicating cathepsin X in the IGF signaling pathways.

To determine if cathepsin X downregulation impairs the expression and activation of IGF receptors, we transfected PC-3 and LNCaP prostate carcinoma cells with small interfering RNAs (siRNAs) targeting cathepsin X as well as with non-targeting control siRNAs and performed quantitative RT-PCR and immunoblot analyses of the corresponding cell lysates. To exclude off-target effects of individual siRNAs, two siRNAs targeting different regions of the cathepsin X mRNA were initially applied, with similar results. For reasons of clarity, only data with one siRNA are shown. Furthermore, to

exclude potential clonal differences, experiments were performed on pools of transiently transfected cells. As determined by quantitative RT-PCR as well as by immunoblotting, the downregulation of cathepsin X mRNA and protein was effective with levels between 80% and 90% when determined 72 h after transfection (Figure 1A and B).

Next, we evaluated the expression levels of growth factor receptors in cathepsin X-deficient prostate carcinoma cells. Quantitative RT-PCR experiments indicated that cathepsin X deficiency is accompanied by an upregulation of IGF-I receptor (IGF-IR) transcripts both in PC-3 and LNCaP cells (Figure 1C and D). In contrast, the expression of the IGF-II receptor (IGF-IIR; cation-independent MPR) and of the cation-dependent MPR were not affected by cathepsin X knockdown in these cells.
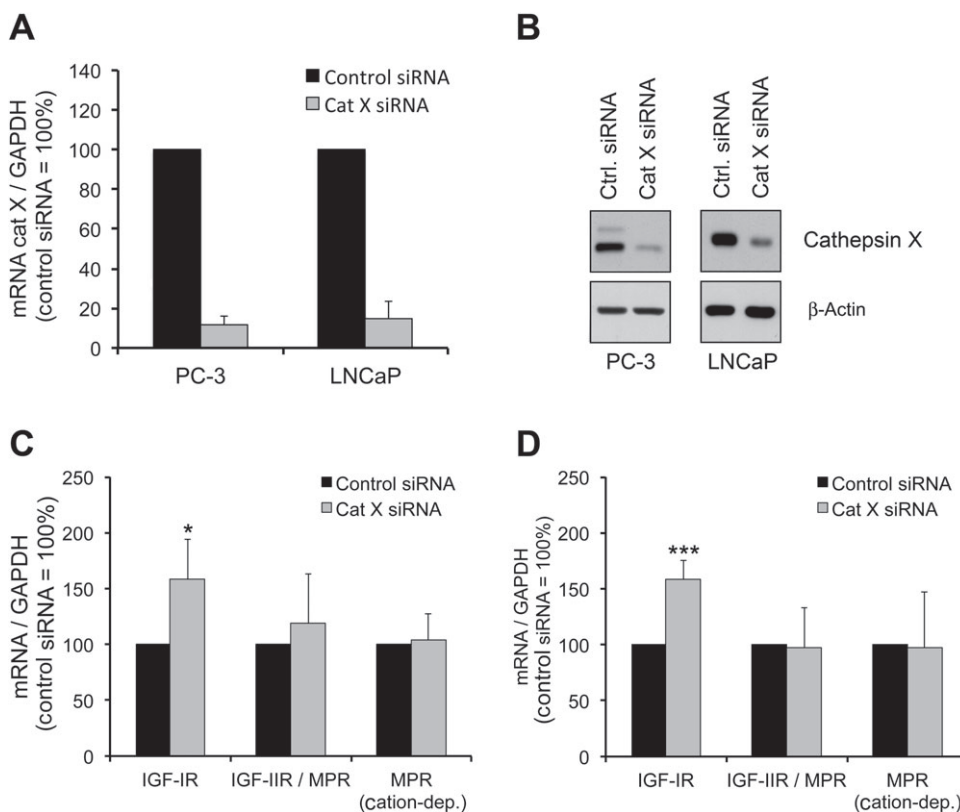

Figure 1 Knockdown of cathepsin X by RNA interference and effects on mRNA levels of IGF receptors.
(A) mRNA transcripts and (B) cell lysates were analyzed by quantitative RT-PCR and immunoblotting 3 days after siRNA transfection as previously described (Kraus et al., 2011). Cathepsin X downregulation after siRNA transfection was confirmed both at (A) the mRNA level and (B) the protein level, revealing knockdown efficiencies of 80%–90%. The abundance of IGF-IR transcripts increases after cathepsin X knockdown in both (C) PC-3 cells and (D) LNCaP cells. In contrast, IGF-IIR/cation-independent MPR expression as well as mRNA levels of the cation-dependent MPR were not altered in cathepsin X-deficient cells. Bars represent means±SD of three independent experiments. Statistical significance was evaluated using Student *t*-test for independent groups. A value of *p*<0.05 was considered statistically significant. *p<0.05, ***p<0.001. Methods: PC-3 or LNCaP prostate carcinoma cells were transfected 3 days before the experiments with siRNAs targeting cathepsin X or with nonsilencing control siRNAs. SiRNAs were synthesized by Qiagen (Hilden, Germany). The sequence of the siRNA targeting cathepsin X was as follows: sense, CGG AUC GGA UCA ACA UCA AdTdT; antisense, UUG AUG UUG AUC CGA UCC GdTdT. All experiments were performed on pools of transiently transfected cells.

To investigate whether cathepsin X-deficient tumor cells display differences in IGF-IR activation, we stimulated PC-3 and LNCaP cells with IGF-I or IGF-II and evaluated the phosphorylation of the IGF-IR using a phosho-specific antibody. Similarly to the mRNA level, unstimulated cells expressed higher protein levels of the IGF-IR after cathepsin X knockdown (Figure 2). Overall, the expression of the IGF-IR was higher in PC-3 cells (Figure 2A) compared with LNCaP cells (Figure 2D), which is in good agreement with data from the literature (Krueckl et al., 2004; Pandini et al., 2005). In cells deficient of cathepsin X, stimulation with IGF-I resulted in reduced phosphorylation compared with control cells. If cells were stimulated with IGF-II, however, IGF-IR phosphorylation increased on an absolute level, but the phospho-IGF-IR/total IGF-IR ratio remained rather unaffected after cathepsin X knockdown (Figure 2C and F).

To further analyze the localization and altered IGF-IR activation in cathepsin X-deficient cells, we performed immunofluorescence on PC-3 cells. The IGF-IR appears to accumulate in smaller vesicles in cells lacking cathepsin X compared with control cells (Figure 3A). After stimulation with IGF-I, the phosphorylated IGR-IR (p-IGF-IR) was detected at the cell border only in control cells, but not after treatment with cathepsin X-specific siRNAs (Figure 3B). Focal adhesion kinase (FAK) is known to interact with the activated IGF-IR and mediates IGF-I signals (Liu et al., 2008; Andersson et al., 2009; Ucar et al., 2011). Co-immunostaining with p-FAK showed a similar staining pattern to the p-IGF-IR at the cell periphery in control cells but not in cathepsin X-deficient cells. In contrast, stimulation with IGF-II did not lead to major differences in staining patterns of both p-IGF-IR and p-FAK, which is in good agreement with the immunoblotting results (data not shown). These results suggest that cathepsin X may mediate IGF-I signaling in a FAK-dependent manner.

Surprisingly, the effects of IGF-I and IGF-II on the proliferation of prostate cancer cells were not significant (data not shown). The cells did not respond to any of the growth factors in a CyQUANT proliferation assay. Instead, the cells were able to grow in serum-free medium, and neither IGF-I nor IGF-II could induce a further increase in proliferation. A possible explanation for this finding is that certain cancer cell lines possess a dramatically deregulated cell cycle leading to uncontrolled proliferation, which is virtually independent from growth factor abundance. A similar observation was reported for bladder cancer cells, which did not respond to IGF-I in terms of proliferation, whereas motility and invasion could be positively influenced by this growth hormone (Metalli et al., 2010). Meanwhile, matching our previous results with
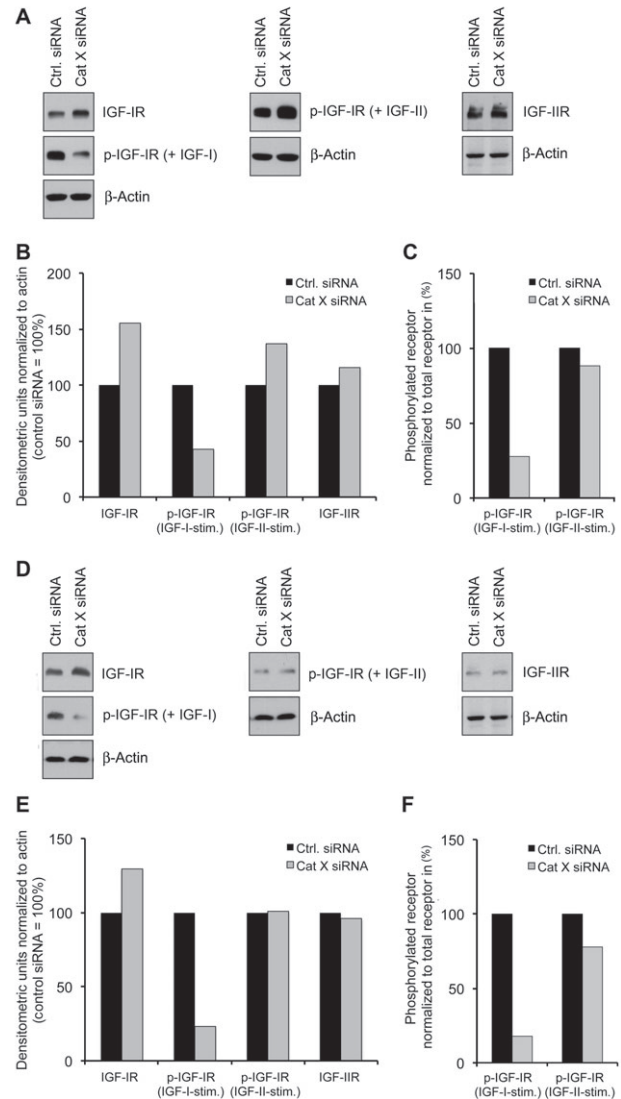

Figure 2 Effects of cathepsin X knockdown on IGF receptor phosphorylation.
(A) Similarly to mRNA levels, total IGF-IR is increased in PC-3 cells lacking cathepsin X, whereas phosphorylated IGF-IR appears to be decreased after stimulation with IGF-I. In contrast, the ratio of phospho-IGF-IR to total IGF-IR does not change after stimulation with IGF-II. The levels of the IGF-IIR were unaffected by cathepsin X siRNA treatment. (B) and (C) Densitometric analysis of immunoblots shown in (A). (D)–(F) Immunoblots and densitometric analysis from similar experiments performed with LNCaP cells. Densitometric analysis was performed using ImageJ (Girish and Vijayalakshmi, 2004). Methods: PC-3 or LNCaP prostate carcinoma cells were transfected 3 days before the experiments with siRNAs targeting cathepsin X or with nonsilencing control siRNAs. Cells were starved for 4 h in serum-free medium and were then stimulated with 50 ng/ml IGF-I or 200 ng/ml IGF-II, respectively. After 20 min (IGF-I) or 10 min (IGF-II) of stimulation, cells were lysed on ice in SDS sample buffer containing Protease Inhibitor Cocktail III (Calbiochem/Merck, Darmstadt, Germany) and 1 mM Na_3VO_4 (Merck, Darmstadt, Germany). Lysates were analyzed by immunoblotting using antibodies for IGF-IR (1:1000; Cell Signaling, Danvers, MA, USA), p-IGF-IR (1:1000; Cell Signaling), IGF-IIR (1:1000; R&D Systems, Minneapolis, MN, USA), and actin (1:500; Sigma-Aldrich, St. Louis, MO, USA).
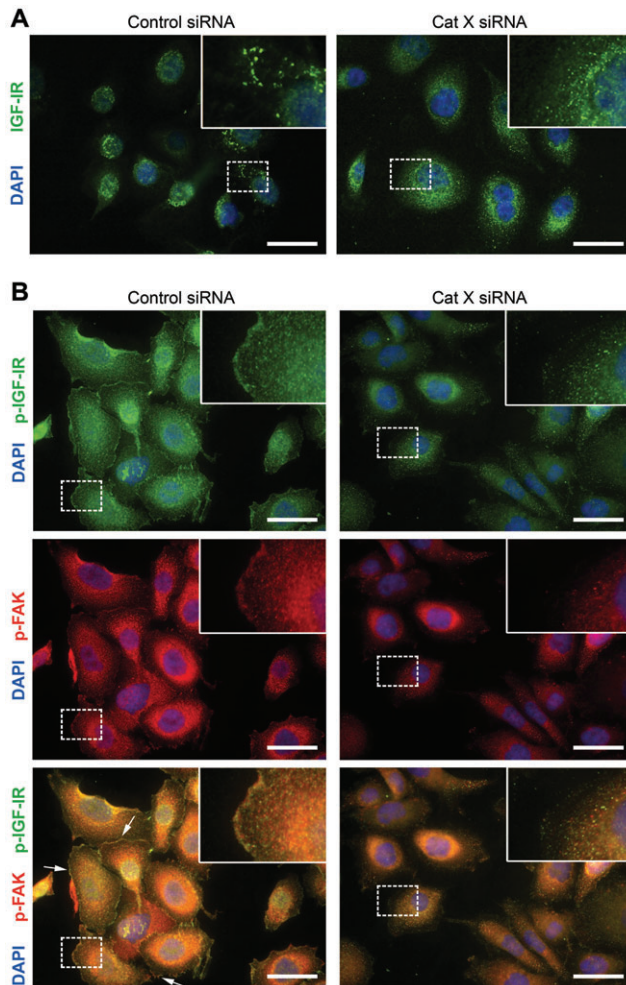
Figure 3 Subcellular localization of the IGF-IR in PC-3 cells.
(A) IGF-IR-positive vesicles are smaller and more evenly distributed in unstimulated cathepsin X-deficient PC-3 cells compared with cells expressing cathepsin X. (B) Upon stimulation with IGF-I, both phosphorylated IGF-IR and phosphorylated FAK appear to be partially co-localized at the cell border in control cells but not in cells treated with cathepsin X siRNAs (arrows). Methods: Three days after transfection with siRNAs, cells were starved for 4 h in serum-free RPMI. Subsequently, IGF-I (50 ng/ml) was added to the cells. After 20 min of stimulation, cells were washed with PBS and fixed in 8% formaldehyde/200 mM HEPES for 30 min at room temperature, washed again with 200 mM HEPES, permeabilized with 0.2% Triton X-100 in PBS followed by washing with PBS. Blocking was performed in 3% BSA/PBS for 30 min at 37°C. Next, samples were incubated with primary antibodies (diluted in 0.1% BSA/PBS) targeting IGF-IR (1:200; Cell Signaling), p-IGF-IR (1:200; Abcam, Cambridge, UK), or p-FAK (1:200; BD Biosciences, Heidelberg, Germany) overnight at 4°C. Incubation with the respective secondary antibody (Alexa Fluor 488 goat anti-rabbit, 1:200; Alexa Fluor 568 goat anti-mouse, 1:200; Molecular Probes/Life Technologies, Darmstadt, Germany) was performed for 1 h at room temperature in 0.1% BSA/PBS. After a final wash, cover slips were mounted with ProLong Gold Antifade Reagent with DAPI (Molecular Probes/Life Technologies). Images were collected on a Carl Zeiss Axioskop upright microscope using a Canon EOS 60D digital camera. Scale bars represent 25 µm.

other cell types, there was a slight decrease in proliferation due to cathepsin X deficiency. With regard to the role of cathepsin X in invasive processes, transwell (Boyden chamber) assays had previously revealed a decreased invasive potential of tumor cells lacking cathepsin X (Krueger et al., 2005). Similarly, cathepsin X-deficient PC-3 cells display a reduction of the invasive capacity in transmigration assays (data not shown).

Taken together, our results indicate that cathepsin X is able to modulate IGF signaling and partly explain our previous findings regarding the accelerated cellular senescence of cathepsin X-deficient cells (Kraus et al., 2011). Increased IGF-IR expression and abundance could be a result of compensatory effects taking into account that receptor activation upon IGF-I stimulation is decreased in cathepsin X-deficient prostate cancer cells. Remarkably, cathepsin X deficiency leads to decreased IGF-IR phosphorylation in response to IGF-I but not IGF-II stimulation. Similarly, downstream signaling events such as phosphorylation of FAK were affected in cathepsin X-deficient PC-3 cells only after stimulation with IGF-I.

In this context, our results suggest that other factors may be involved in IGF-II-induced signaling. IGF-II can bind with high affinity to both the IGF-I and the IGF-II/cation-independent MPR. The IGF-IIR is not involved in growth-promoting signal transduction pathways; however, it is thought to act as a sink for IGF-II, thereby preventing this growth factor to activate the IGF-IR (Ellis et al., 1996). Meanwhile, extracellular cathepsins are also able to interact with the IGF-II receptor *via* their M6P moiety. As a consequence, the enzyme-receptor complexes are internalized, leading to a decrease of the number of receptor molecules at the cell surface. As cathepsin X is the main lysosomal enzyme secreted from prostate cancer cells (data not shown), its reinternalization through its interaction with the IGF-II/cation-independent MPR may drive extracellular IGF-II molecules to activate the IGF-IR, thereby inducing IGF signaling. In turn, cathepsin X downregulation may lead to an increased internalization of IGF-II and thereby contribute to impaired IGF signaling. Taking into account that both PC-3 and LNCaP cells produce endogenous IGF-II, this model is supported by our finding that cathepsin X deficiency leads to reduced activation of the IGF-IR in response to IGF-I.

Hence, these results reflect the complexity of the underlying mechanism. As cathepsin X deficiency does not induce changes in IGF-II-dependent IGF-IR phosphorylation, cathepsin X appears to act mainly on the IGF-I axis.

Cathepsin X could also be involved in the processing of IGF-binding proteins, thereby disturbing the interaction

between these and IGF-I and leading to a higher bioavailability of IGF-I. Furthermore, recent evidence on the crosstalk between the IGF-IR and the integrins adds a new level of complexity to cellular signaling (Beattie et al., 2010). One could, therefore, also speculate on the interference of cathepsin X with the crosstalk between the IGF axis and the integrins because cathepsin X has been shown to physically interact with integrins (Lechner et al., 2006; Jevnikar et al., 2011).

Remarkably, a loss of responsiveness to IGF-I was also observed in cells with reduced cathepsin L expression levels; however, cathepsin L downregulation led to increased apoptosis rather than senescence (Navab et al., 2008). Therefore, the influence of cathepsins on IGF signaling is likely to be widespread and complicated,

and further research will continue to advance our knowledge regarding these mechanisms. In particular, the interference of cathepsin X with IGF signaling in normal, untransformed cells will reveal whether this mechanism is confined to tumor cells or cathepsin X and/or other cathepsins interfere with IGF signaling during normal regulation of growth-promoting pathways.

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