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# Concanavalin-A-induced autophagy biomarkers requires membrane type-1 matrix metalloproteinase intracellular signaling in glioblastoma cells

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**Pre-clinical trials for cancer therapeutics support the anti-neoplastic properties of the lectin from *Canavalia ensiformis* (Concanavalin-A, ConA) in targeting apoptosis and autophagy in a variety of cancer cells. Given that membrane type-1 matrix metalloproteinase (MT1-MMP), a plasma membrane-anchored matrix metalloproteinase, is a glycoprotein strongly expressed in radioresistant and chemoresistant glioblastoma that mediates pro-apoptotic signalling in brain cancer cells, we investigated whether MT1-MMP could also signal autophagy. Among the four lectins tested, we found that the mannopyranoside/glucopyranoside-binding ConA, which is also well documented to trigger MT1-MMP expression, increases autophagic acidic vacuoles formation as demonstrated by Acridine Orange cell staining. Although siRNA-mediated MT1-MMP gene silencing effectively reversed ConA-induced autophagy, inhibition of the MT1-MMP extracellular catalytic function with Actinonin or Ilomastat did not. Conversely, direct overexpression of the recombinant Wt-MT1-MMP protein triggered proMMP-2 activation and green fluorescent protein–microtubule-associated protein light chain 3 puncta indicative of autophagosomes formation, while deletion of MT1-MMP's cytoplasmic domain disabled such autophagy induction. ConA-treated U87 cells also showed an upregulation of BNIP3 and of autophagy-related gene members autophagy-related protein 3, autophagy-related protein 12 and autophagy-related protein 16-like 1, where respective inductions were reversed when MT1-MMP gene expression was silenced. Altogether, we provide molecular evidence supporting the pro-autophagic mechanism of action of ConA in glioblastoma cells. We also highlight new signal transduction functions of MT1-MMP within apoptotic and autophagic pathways that often characterize cancer cell responses to chemotherapeutic drugs.**

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## Introduction

The expression level of numerous members of the matrix metalloproteinase (MMP) family has been shown to correlate with the grade level of gliomas, including that of the membrane type-1 (MT1)-MMP (Pagenstecher et al. 2001). Most MMPs are produced and released outside the cell; however, the MT-MMPs are anchored to the cell membrane and a number of them possess a cytoplasmic domain recognized to contribute to important intracellular signaling functions (Gingras et al. 2001; Li et al. 2008). More importantly, glioma-associated microglia has been shown to induce and exploit MT1-MMP expression for tumor expansion, while MT1-MMP overexpression in the glioma cells was rather found lethal (Markovic et al. 2009). Besides its well-known roles in the activation of proMMP-2 and in intrinsic proteolytic activity toward extracellular matrix molecules, many other functions have been assigned to MT1-MMP. In recent years, fundamental new roles of MT1-MMP have been documented in bioactive lisophospholipid signaling (Annabi, Lachambre et al. 2009), nuclear factor-kappa B (NF-κB)-mediated cyclooxygenase (COX)-2 regulation (Han et al. 2001; Annabi, Laflamme et al. 2009; Sina et al. 2010), radioresistance in both glioma (Wild-bode et al. 2001; Wick et al. 2002) and endothelial cells (Annabi et al. 2003), and as a cell death sensor/effector (Belkaid et al. 2007; Currie et al. 2007; Proulx-Bonneau, Guezguez et al. 2011; Proulx-Bonneau, Pratt et al. 2011). More importantly, a role for MMPs in autophagic cell death has been suggested (Augustin et al. 2009), but the exact mechanisms and their biological significance in this process remain poorly understood.

Current conventional cancer therapies fail to mediate their effects in a target-specific fashion (Quant et al. 2010; Sampson et al. 2011). Among the reasons, the extremely unfavorable prognosis for patients suffering from glioblastomas was strongly correlated to inefficient targeting of their intrinsic apoptosis-resistant phenotype. Besides apoptosis-based therapies, induction of autophagic cell death is becoming an alternative and emerging concept to trigger glioma cell death and to exploit caspase-independent programmed cell death pathways for the development of novel glioma therapies (Kogel et al.

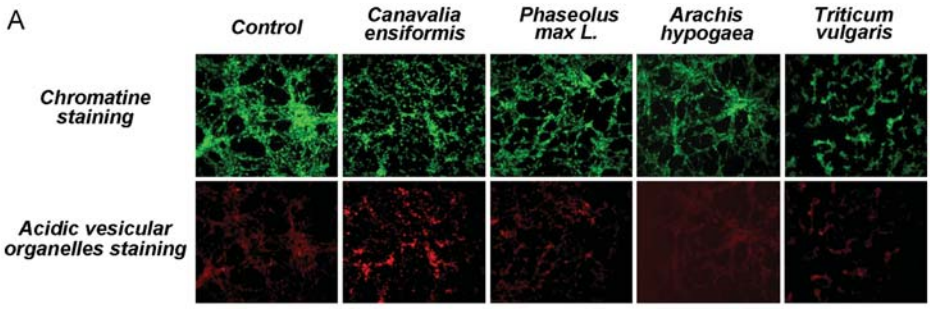
2010). Induction of autophagic cell death may therefore help stop tumor development and optimize cancer treatment modalities (Ceteci et al. 2011; Guo et al. 2011; Chen et al. 2012). Interestingly, recent evidence indicates the clinically approved antibiotic minocycline, a highly lipid-soluble antibacterial known for its superior ability to cross the blood-brain barrier, to act as a promising new candidate for adjuvant therapy against malignant gliomas by reducing MT1-MMP expression (Markovic et al. 2011). More importantly, minocycline effectively inhibited tumor growth and induced autophagy in a xenograft tumor model of C6 glioma cells (Liu, Lin, Yu et al. 2011; Liu, Lin, Hsiao et al. 2011). Whether an intrinsic role can thereof be attributed to MT1-MMP in autophagy processes remains unknown.

Numerous cellular recognition processes including development, differentiation, morphogenesis and cell migration are regulated upon interactions between carbohydrate-binding proteins (lectins) and cell surface oligosaccharide moieties of glycoproteins (Kohn et al. 2004). In the present study, we used the lectin from *Canavalia ensiformis* (Concanavalin-A, ConA), commonly used to trigger both MT1-MMP-mediated activation of latent proMMP-2 (Lin and Levitan, 1991; Yu et al. 1997; Zucker et al. 2002; Lafleur et al. 2006), and cell death events (Wild-Bode et al. 2001). Given ConA has been shown to bear potential anti-neoplastic properties (Lei and Chang, 2009; Li, Zhu et al. 2011; Li, Yu et al. 2011), we assessed the role of MT1-MMP in the ConA-mediated signaling of autophagy processes. We show through structure–function studies the direct requirement of the intracellular domain of recombinant MT1-MMP constructs in autophagy induction. Differential autophagy-related gene arrays enabled us to further link MT1-MMP-mediated signaling to BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3) and to identify autophagy-related genes autophagy-related protein 3 (ATG3), autophagy-related protein 12 (ATG12) and autophagy-related protein 16-like 1 (ATG16-L1) expressions among candidates involved in the autophagic cell death of glioblastoma cells.

## Results

### *High cell surface mannopyranoside residues contribute to lectin-induced autophagy*

Given the documented cell death-inducing potential of plant lectins (Fu et al. 2011), we further tested their potential to trigger autophagy. Serum-starved U87 glioblastoma cells were treated with the following four lectins: *C. ensiformis*, *Phaseolus max* L., *Arachis Hypogaea* and *Triticum vulgaris*, and then were stained with Acridine Orange. While chromatin staining remained unchanged (Figure 1A, upper panels in green), we observed that significant acidic vesicular organelle (AVO) formation (Figure 1A, lower panels in red) was specifically associated with ConA (*C. ensiformis*). Given the lectin-specific and distinctive cell surface carbohydrate moieties interaction potential (Figure 1B), one can safely speculate that such autophagy induction may necessitate high mannosyl residues, and that AVO formation correlates with autophagosome formation as visualized through electronic microscopy of untreated and ConA-treated cells (Figure 1C).




B

| Nomenclature | Origin | Carbohydrate specificity |
|---|---|---|
| ConA | *Canavalia ensiformis* | High-Man, Manα1-6(Manα1-3)Man |
| SBA | *Phaseolus max L.* | α- or β-linked terminal GalNAc, GalNAcα1-3Gal |
| PNA | *Arachis hypogaea* | Galβ1-3GalNAc |
| WGA | *Triticum vulgaris* | Chitin oligomers, NeuAc |

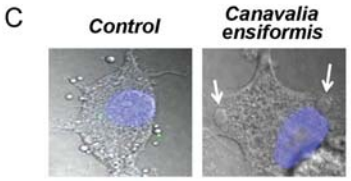



**Fig. 1.** High cell surface mannopyranoside residues contribute to lectin-induced autophagy. Serum-starved U87 were treated for 24 h with 30 μg/mL of the following four lectins: *C. ensiformis*, *Phaseolus max* L., *Arachis Hypogaea*, and *Triticum vulgaris*. (**A**) Chromatin staining (green) and AVOs staining (red) was performed. (**B**) Description of the carbohydrate specificity for each lectin tested. (**C**) Electronic microscopy pictures of autophagosomes formation (white arrows). Nucleus staining was performed with DAPI attaining (blue).

*ConA triggers proMMP-2 activation and autophagy*

ConA is a well-documented lectin which, through its binding to carbohydrate moieties on cell surface glycoproteins, elicits very efficient in vitro induction of MT1-MMP expression (Fortier et al. 2008; Annabi, Laflamme et al. 2009; Sina et al. 2010). Serum-starved U87 glioblastoma cells were therefore treated with increasing concentrations of ConA and then stained with Acridine Orange. Images of U87-treated cells were taken using fluorescent microscopy as described in the Methods section. ConA was found to dose-dependently increase the production of AVOs (Figure 2A). Then, proMMP-2 activation into MMP-2 was assessed by gelatin zymography in response to increasing concentrations of ConA (Figure 2B). When proMMP-2 activation (MMP-2/proMMP-2 ratio) was plotted against Acridine Orange staining intensity, a positive linear correlation ($r^2 = 0.98$) was observed (Figure 2C). Treatment of serum-starved U87 glioblastoma cells with ConA for up to 24 h revealed increasing production of AVOs, whereas no effect was observed in untreated control cells (Figure 2D).

*ConA-induced autophagy is independent of MT1-MMP catalytic function*

A structure–function study was next used to address whether MT1-MMP's catalytic domain is responsible for the ConA-induced autophagy. U87 cells were treated with Ilomastat, a broad range MMP inhibitor known to target MT1-MMP extracellular domain catalytic functions, or Actinonin, an aminopeptidase N/CD13 inhibitor known to target MT1-MMP-mediated proMMP-2 activation (Sina et al. 2009), and stained with Acridine Orange. We show that Ilomastat and Actinonin were unable to reverse the effect of ConA-induced autophagy (Figure 3A and B), although both efficiently inhibited MT1-MMP-mediated proMMP-2 activation into MMP-2 (Figure 3C). These results suggest that MT1-MMP's extracellular catalytic domain is not involved in the induction of autophagy.

*MT1-MMP gene silencing antagonizes ConA-induced autophagy*

ConA is well known to trigger autophagy (Chang et al. 2007) and MT1-MMP expression (Yu et al. 1997). In order to delineate the specific contribution of MT1-MMP to ConA-induced autophagy, gene silencing was performed using either a scrambled sequence of siRNA (siScrambled) or an siRNA directed against MT1-MMP gene expression (siMT1-MMP). We observed that serum-starved U87 glioblastoma cells treated with ConA triggered MT1-MMP gene expression in both Mock and siScrambled conditions (Figure 4A). In contrast, siMT1-MMP-transfected cells invalidated ConA's ability to trigger MT1-MMP gene expression. MT1-MMP gene silencing also abrogated the functional role of MT1-MMP in ConA-induced proMMP-2 activation into MMP-2 as assessed

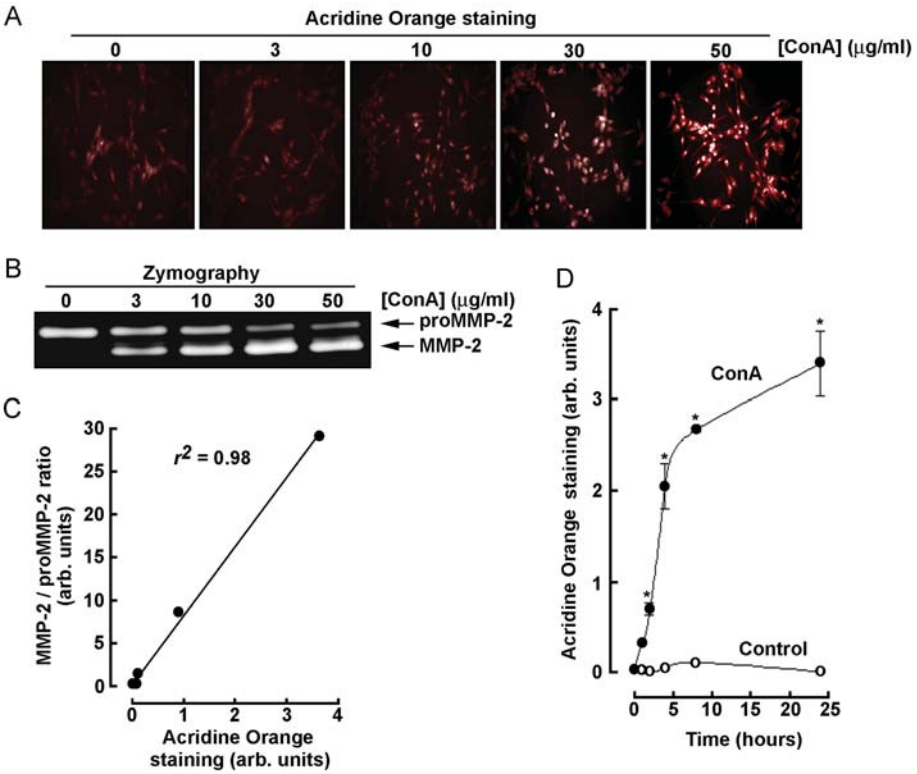



**Fig. 2.** Concanavalin-A (ConA) triggers proMMP-2 activation and autophagy. Serum-starved U87 glioblastoma cells were treated with increasing concentrations of ConA for 24 h. (**A**) Cells were stained with Acridine Orange as described in the Methods section and acidic vacuole formation examined by fluorescent microscopy. (**B**) Conditioned media were isolated and gelatin zymography performed to assess the extent of proMMP-2 activation into MMP-2. (**C**) Scanning densitometry was performed from the zymogram and correlations plot made between MMP-2/proMMP-2 ratios and Acridine Orange staining. (**D**) Acridine Orange staining was quantified in control (open circles) and 30 μg/mL ConA-treated (closed circles) U87 glioblastoma cells for different time points.

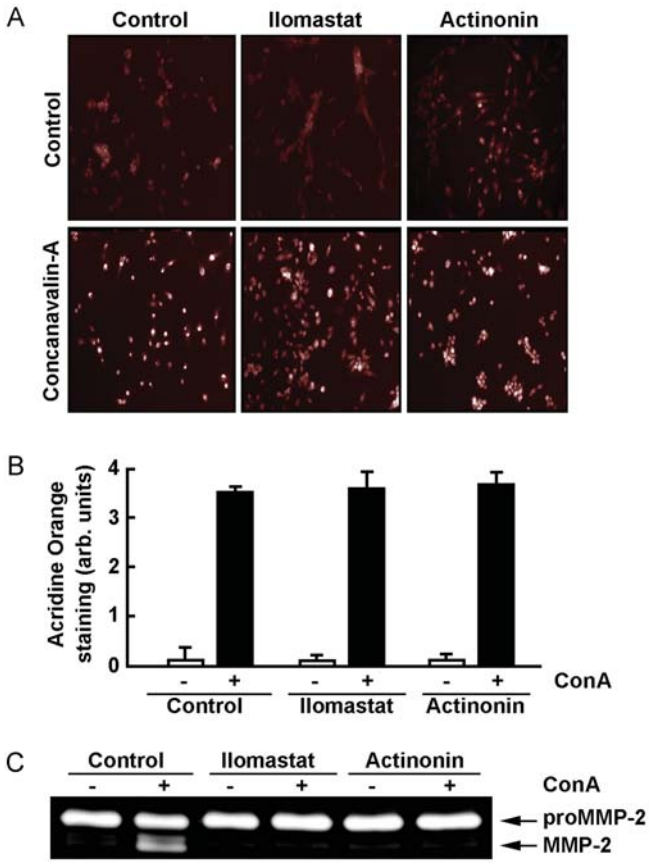



**Fig. 3.** ConA-induced autophagy is independent of MT1-MMP extracellular catalytic function. Serum-starved U87 glioblastoma cells were treated with vehicle or 30 μg/mL ConA, 25 μM Ilomastat, 30 μM Actinonin or a combination of ConA with either Ilomastat or Actinonin for 24 h. (**A**) Cells were stained with Acridine Orange as described in the Methods section, and fluorescence examined by microscopy. (**B**) Scanning densitometry was performed as shown in (A) in order to assess the extent of acidic vacuoles formation in control (white bars) and ConA-treated cells (black bars). (**C**) Conditioned media were isolated and gelatin zymography performed to assess the extent of proMMP-2 activation into MMP-2.

by gelatin zymography (Figure 4B). Staining for AVOs with Acridine Orange (Figure 4C) revealed that MT1-MMP gene silencing efficiently abrogated ConA's ability to trigger autophagy (Figure 4D). Collectively, this suggests that MT1-MMP is an important contributor to ConA-mediated autophagy.

### *The intracellular domain of MT1-MMP is required to signal autophagy*

Given that MT1-MMP is important for ConA-induced autophagy and that we excluded MT1-MMP's extracellular domain functions, we then assessed the direct contribution of MT1-MMP in autophagy induction. More specifically, the design of structure–function approaches, using recombinant MT1-MMP constructs, was performed using cDNA encoding either full length Wt-MT1-MMP, a cytoplasmic-deleted (Δ-Cyto)-MT1-MMP, or a cytoplasmic domain and transmembrane domain (Δ-TM) soluble MT1-MMP. Transient transfections were performed in U87 glioblastoma cells which were

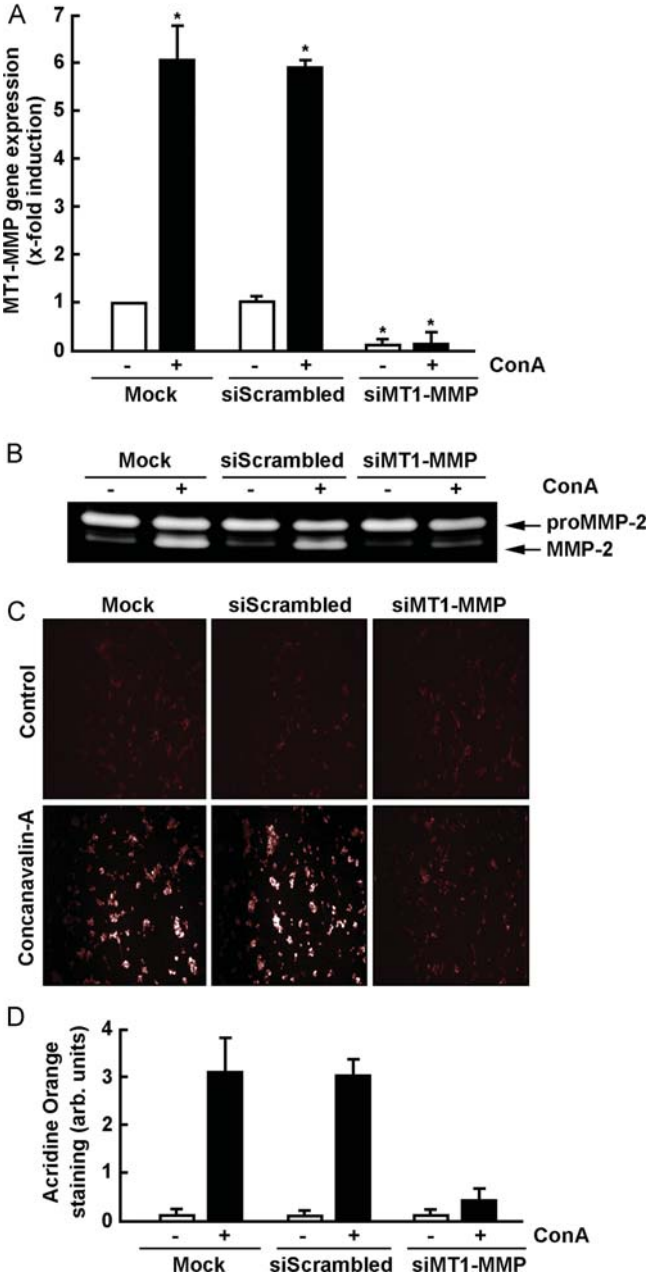



**Fig. 4.** Gene silencing of MT1-MMP gene expression antagonizes ConA-induced autophagy. Gene silencing was performed with either siScrambled or siMT1-MMP in U87 glioblastoma cells, or with lipofectamine treatment only (Mock). Cells were then serum-starved in the presence (black bars) or absence (white bars) of 30 μg/mL ConA. (**A**) Total RNA was isolated and qRT-PCR performed as described in the Methods section to assess MT1-MMP gene expression. (**B**) Conditioned media were isolated and gelatin zymography performed to assess the extent of proMMP-2 activation into MMP-2. (**C**) Cells were stained with Acridine Orange as described in the Methods section, and fluorescence examined by microscopy. (**D**) Scanning densitometry was performed as shown in (C) in order to assess the extent of acidic vacuoles formation in control (white bars) and ConA-treated cells (black bars).

subsequently serum starved. Acridine Orange staining (Figure 4A) revealed that only the Wt-MT1-MMP overexpression enabled autophagy, while the Δ-Cyto-MT1-MMP and Δ-TM-MT1-MMP forms had no effect (Figure 5B). The

respective conditioned media and cell lysates were used to validate the overexpression and function of the overexpressed recombinant proteins. Only Wt-MT1-MMP and Δ-Cyto-MT1-MMP were able to constitutively activate proMMP-2 (Figure 5C, upper panel), while only the Δ-TM-MT1-MMP was detected in the conditioned media (Figure 5C, second panel). Green fluorescent protein-light chain 3 (pEGFP-LC3) puncta was also used to assess autophagosome formation. Cells were co-transfected with a plasmid encoding pEGFP-LC3 combined with either Wt-MT1-MMP, Δ-Cyto-MT1-MMP or Δ-TM-MT1-MMP cDNA plasmids as described above. Significant puncta, indicative of autophagosome formation, were only observed within cells overexpressing Wt-MT1-MMP or cells that were treated with ConA (Figure 5D).

### *ConA-induced autophagy triggers specific MT1-MMP-mediated autophagy-related genes*

In light of the evidence that MT1-MMP contributes to ConA-mediated autophagy, we used a gene array approach to identify ConA-induced autophagy gene targets. Transcriptional profiling was performed and, among the 89 autophagy and autophagosome formation-related genes (Itakura and Mizushima 2010), we found BNIP3, ATG3, ATG12 and ATG16-L1 to be upregulated, while ATG16L2 gene expression was downregulated (Figure 6, left panel). When ConA treatment was performed in siMT1-MMP-transfected cells, expression of these four genes returned to basal levels (Figure 6, right panel). Specific contribution of MT1-MMP in the transcriptional regulation of those four genes was further confirmed in transfected cells. A single cDNA amplicon was found when quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed for BNIP3, ATG3, ATG12 and ATG16L1 (Figure 7A). Only Wt-MT1-MMP-enabled gene expression increases, while the Δ-Cyto-MT1-MMP remained ineffective (Figure 7B). In conclusion, BNIP3 and the ATG gene members 3, 12 and 16L1 can therefore be considered under MT1-MMP-mediated transcriptional control in ConA-treated cells.

## Discussion

There are accumulating evidences implicating autophagy in cancer, neurodegenerative diseases, infectious as well as cardiovascular diseases (Kroemer and Levine, 2008; Mizushima

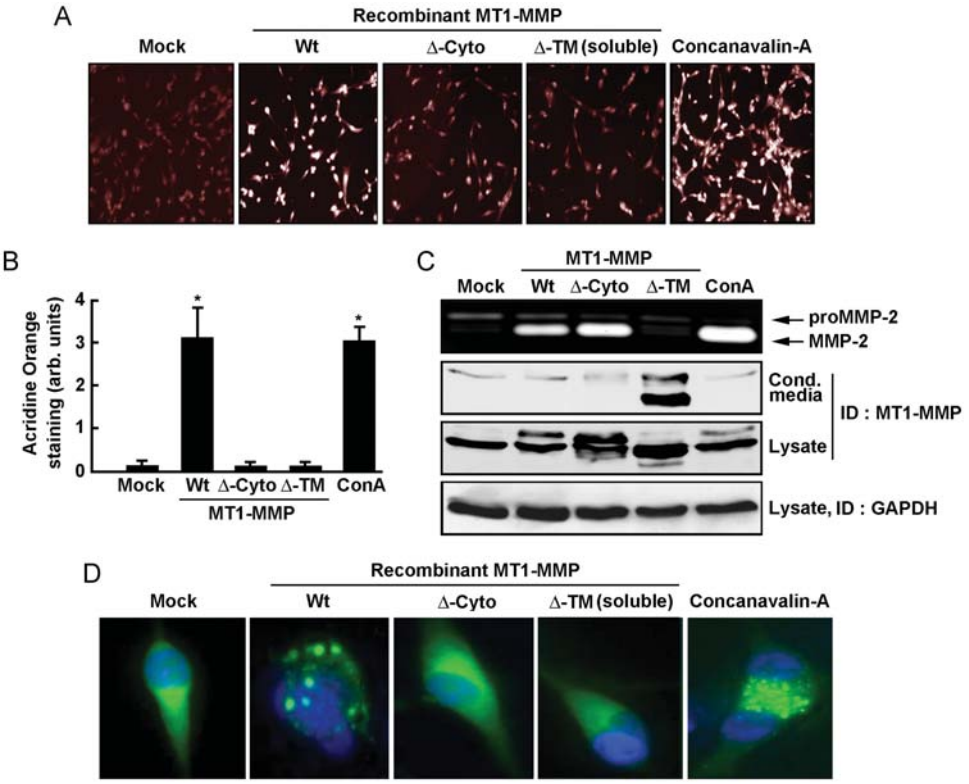



**Fig. 5.** The intracellular cytosolic domain of MT1-MMP is required to trigger autophagy. U87 glioblastoma cells were transiently transfected with cDNA plasmids encoding either full-length Wt-MT1-MMP, a cytoplasmic-deleted (Δ-Cyto)-MT1-MMP, a cytoplasmic domain and transmembrane domain (Δ-TM) soluble MT1-MMP or empty pcDNA (Mock). (**A**) Serum-starved cells were stained with Acridine Orange as described in the Methods section and (**B**) scanning densitometry performed on the pictures in order to assess the extent of acidic vacuoles formation. (**C**) Conditioned media as well as cell lysates were isolated as described in the Methods section in order to perform gelatin zymography (top) or to perform MT1-MMP immunodetection in the conditioned media (second from top) and in the lysates (second from bottom) and GAPDH (bottom). (**D**) Photomicrographs showing the intracellular distribution of exogenous LC3. Cells were transiently co-transfected with a pEGFP-LC3-expressing plasmid and either Wt-MT1-MMP, Δ-Cyto-MT1-MMP, Δ-TM-MT1-MMP or empty pcDNA (control), or treated with ConA. Samples were fixed 24 h later and nuclei counterstained with Hoechst 33342. Characteristic punctates of pEGFP-LC3 staining can be observed in Wt–MT1-MMP-transfected and ConA-treated cells.

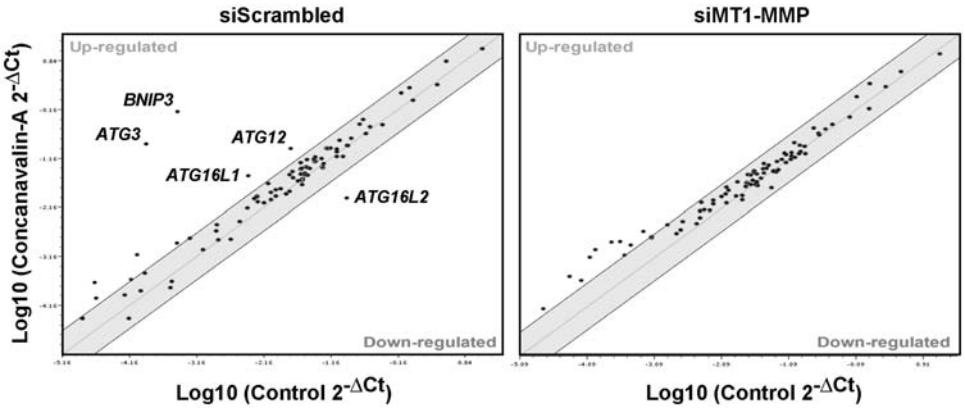



**Fig. 6.** ConA-induced autophagy-related genes are reduced in siMT1-MMP transfected U87 cells. Gene silencing was performed with either siScrambled or siMT1-MMP in U87 glioblastoma cells. Cells were then serum-starved in the presence or absence of 30 µg/mL ConA for 24 h. Total RNA isolation and qRT-PCR were performed as described in the Methods section to assess expression of a subset of 84 different autophagy-related genes using a human autophagy PCR array.

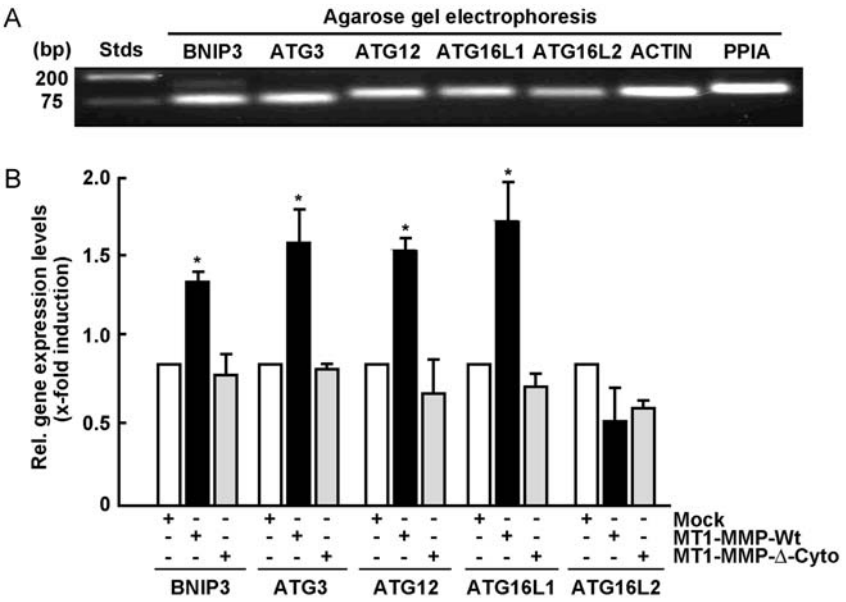



**Fig. 7.** Transcriptional regulation of BNIP3, ATG3, ATG12, ATG16L1 and ATG16L2 expression is signaled through MT1-MMP's intracellular domain. U87 glioblastoma cells were transiently transfected with cDNA plasmids encoding either full-length Wt-MT1-MMP, a cytoplasmic-deleted (Δ-Cyto)-MT1-MMP, or empty pcDNA (Mock). Cells were then serum-starved for 24 h. Total RNA isolation and qRT-PCR were performed as described in the Methods section. (**A**) Amplicon for each gene were loaded onto an agarose gel to show BNIP3-, ATG3-, ATG12-, ATG16L1- and ATG16L2-specific amplification. (**B**) Gene expressions in Wt–MT1-MMP- (black bars) and ΔCyto-MT1-MMP-transfected cells (grey bars) were compared with that of Mock (white bars)-transfected cells.

et al. 2008). Although the specific roles of MMP in autophagy still remain to be unraveled, chemical modulators of autophagy such as the selective MMP-2 inhibitor ARP101 (Jo et al. 2011) seem to offer some potential for treatment of these diseases although the precise molecular mechanism of action remains unknown. Interestingly, we recently reported the molecular mechanism of action of Brefeldin-A, another well-known autophagy regulator, through sequestration of MT1-MMP and induction of endoplasmic reticulum (ER) stress (Proulx-Bonneau, Guezguez et al. 2011; Proulx-Bonneau, Pratt et al. 2011). This new role of a membrane-bound MMP in transducing Brefeldin-induced ER stress signaling is supported by the emerging data which indicate that ER stress is also a potent inducer of macroautophagy (Matus et al. 2008; Yin et al. 2012). It is currently debated whether such process either enhances cell survival or commits cells to nonapoptotic death (Høyer-Hansen and Jäättelä 2007).

In cancer cells, induction of autophagy serves as an adaptive response that can lead to chemoresistance mechanisms and increased cell survival (Reuter et al. 2010); many of the features also associated with high cellular MT1-MMP levels (Trog et al. 2006). Thus, the inhibition of autophagy

combined with inducers of metabolic stress or chemotherapeutic agents could enhance effective anticancer therapy by inhibiting stress adaptation and increasing cell killing. Pharmacological approaches have demonstrated that diet-derived epigallocatechin gallate, a polyphenol shown to trigger autophagy (Li, Yu et al. 2011; Li, Zhu et al. 2011) and to sensitize cells to radiation (McLaughlin et al. 2006), as well as mammalian target of rapamycin inhibitors such as rapamycin, can be used to increase the radiosensitivity of glioblastoma cells by the induction of autophagy (Zhuang et al. 2009). In support to a possible role for MT1-MMP in intracellular transduction event regulation, rapamycin was recently shown to upregulate MT1-MMP expression in phosphatase and tensin homolog (PTEN) (+/+) cells via PI3K activity (Kim et al. 2010). Tumor suppressors such as Beclin-1, PTEN and p53 are also crucial players in the induction and regulation of autophagy (Liu, Lin, Hsiao et al. 2011; Liu, Lin, Yu et al. 2011). Our current study in fact provides evidence that MT1-MMP's intracellular domain is an absolute requirement for lectin-induced autophagy. Such evidence is further supported by the demonstration that failure of MT1-MMP to localize at the plasma membrane trigger ER stress and abrogated COX-2 expression (Proulx-Bonneau, Guezguez et al. 2011; Proulx-Bonneau, Pratt et al. 2011). Induction of ER stress was also abrogated when either MT1-MMP expression was silenced or the mature 60 kDa form of MT1-MMP was trapped within the ER (Proulx-Bonneau, Guezguez et al. 2011; Proulx-Bonneau, Pratt et al. 2011). As a consequence in tumorigenic processes, it is hypothesized that MT1-MMP may become either active inside cancer cells prior to its presentation at the cell surface or to transduce some crucial signaling once anchored to the plasma membrane. Whether combined regulation of pericellular proteolysis and cleavage of crucial intracellular co-compartmentalized substrate proteins also involves MT1-MMP-mediated autophagic processes remains to be determined. Finally, lectin-targeted cell surface receptor-type glycoproteins often induce their intracellular signaling once oligomerized (Rozanov et al. 2001; Lehti et al. 2002). How such a phenomenon occurs in ConA-mediated MT1-MMP intracellular signaling mechanism still remains speculative. It is thought that MT1-MMP glycosylation status affects its substrate-targeting properties (Wu et al. 2004) and autolysis (Remacle et al. 2006). Whether such MT1-MMP oligomerization occurs upon ConA treatment was however not observed in our experimental conditions.

Generation of nutrients and energy in response to starvation or other metabolic stress conditions is observed in tumor cells upon autophagy processes in order to promote cell survival. In response to numerous stress conditions, autophagy is therefore able to protect dormant cells so that to resume growth in more favorable conditions (Mathew et al. 2007). The diffuse-infiltrative phenotype of gliomas into normal brain tissue of patients (Glioma Meta-analysis Trialists (GMT) Group 2002), combined with the recently reported presence of glioma-initiating cells, may all be conditions that favor the initiation and recurrence of glioblastoma (Zhuang et al. 2011). Accordingly, autophagy was also shown to play an essential role in the regulation of glioma-initiating cells tumorigenic potential (Zhuang et al. 2011). Given that groups currently defining staging for neoplasms assess and incorporate measures of the presence of apoptosis, autophagy and necrosis (Demaria et al. 2010), autophagy could be considered as a promising therapeutic target in specifically defined subsets of glioblastomas. As such, the documented anti-proliferative and anti-tumor activities of ConA against a variety of cancer cells have been reported, where numerous signaling axis have been shown to transduce some of its effects through IκappaB kinase-NF-κB-COX-2, SH2-domain containing phosphatase (SHP)-2-MEK-1-ERK and SHP-2-Ras-ERK (Li, Yu et al. 2011; Li, Zhu et al. 2011). Interestingly, evidence that MT1-MMP also activates a number of common intracellular signal pathways including the extracellular signal-regulated kinases (ERK) pathway, the focal adhesion kinase, Src, RhoA/ROK, Rac and NF-κB pathways has been reported (Takino et al. 2004; Annabi et al. 2005; Sato et al. 2005; Sounni and Noel 2005; Annabi, Laflamme et al. 2009). More importantly, numerous scaffolding and intracellular signaling proteins have also been found to be recruited and to interact with MT1-MMP's intracellular domain. Among others, these include gC1qR (Rozanov et al. 2002), Caveolin-1 (Labrecque et al. 2004), MT1-MMP cytoplasmic tail binding protein (Hirano et al. 2005), actin-cortactin (Artym et al. 2006), the radixin FERM domain (Terawaki et al. 2008), 3BP2 (Proulx-Bonneau, Guezguez et al. 2011; Proulx-Bonneau, Pratt et al. 2011) and the adaptor protein p130Cas (Gingras et al. 2008). Although structural evidence for the transducing impact of the $^{571}$LLY$^{573}$ intracellular domain of MT1-MMP has been provided, combined with transcriptional regulation evidence of vascular endothelial growth factor (Sounni et al. 2004), Smad1 (Freudenberg and Chen 2007) or tumor suppressor Dickkopf-3 (Saeb-Parsy et al. 2008), it is yet to be further documented on how autophagy biomarkers transcriptional regulation is performed by MT1-MMP.

Our current data also highlight, for the first time, an MT1-MMP-mediated transcriptional regulation of autophagy biomarkers BNIP3, ATG3, ATG12, ATG16L1, ATG16L2 upon ConA treatment. This was demonstrated indirectly through a differential gene array approach (Figure 5), and confirmed directly upon overexpression of the Wt–MT1-MMP recombinant protein (Figure 6). Given the recent report that BNIP3 acts as transcriptional repressor of apoptosis-inducing factor expression and prevents cell death in human malignant gliomas (Burton et al. 2009), our data further link the possible contribution of MT1-MMP to the radioresistance and chemotherapeutic resistance index of brain tumors. In fact, BNIP3 is expressed at high levels in solid tumors, including glioblastoma, where its nuclear location is believed to confer a survival advantage to glioma cells (Burton et al. 2006). BNIP3 upregulation under hypoxic conditions by the transcription factor Hypoxia-inducible factor 1 remains open for debate (Namas et al. 2011; Zhao et al. 2012), although demonstrated to locate within hypoxic regions of tumors (Sowter et al. 2001). Given the recent MT1-MMP demonstrated role in hypoxia-regulated events (Proulx-Bonneau and Annabi 2011; Proulx-Bonneau, Guezguez et al. 2011; Proulx-Bonneau, Pratt et al. 2011), it becomes tempting to assume possible similar events taking place in the

MT1-MMP-mediated regulation of BNIP3 in autophagy. Altogether, these biological findings shed light on new perspectives of ConA as a potential antineoplastic agent targeting apoptosis, autophagy and antiangiogenesis in preclinical or clinical trials for cancer therapeutics.

## Materials and methods

### *Materials*

Protein assays were performed with the microbicinchoninic acid protein assay reagents (Pierce, 23 235), and standard curves were generated with bovine serum albumin (BSA; Calbiochem, 2980). All lectins were purchased from Sigma-Aldrich, Canada.

### *Cell culture*

The human U87 glioblastoma cell line (American Type Culture Collection, HTB-14) was maintained in Eagle's Minimum Essential Medium (Wisent, 320-006CL) containing 10% (v/v) calf serum (HyClone Laboratories, SH30541.03), 1 mM sodium pyruvate (Sigma-Aldrich, P2256), 2 mM glutamine (Gibco, 25030), 100 units/mL penicillin and 100 mg/mL streptomycin (Wisent, 250-202-EL). Cells were incubated at 37°C with 95% air and 5% $CO_2$.

### *Total RNA isolation, cDNA synthesis and real-time qRT-PCR*

Total RNA was extracted from cell monolayers using TriZol reagent (Life Technologies, 15596-018). For cDNA synthesis, 2 µg of total RNA were reverse-transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, 4368814). cDNA was stored at −80°C prior to PCR. Gene expression was quantified by real-time quantitative PCR using iQ SYBR Green Supermix (Bio-Rad, 170-8884). DNA amplification was carried out using an Icycler iQ5 (Bio-Rad) and product detection was performed by measuring binding of the fluorescent dye SYBR Green I to double-stranded DNA. The QuantiTect primer sets were provided by QIAGEN : MT1-MMP (Hs_Mmp14_1_SG QT00001533), BNIP3 (Hs_BNIP3_1_SG QT00024178), ATG3 (Hs_ATG3_1_SG QT00069769), ATG12 (Hs_ATG12_1_SG QT00035854), ATG16L1 (Hs_ATG16L1_1_SG QT00085442), ATG16L2 (Hs_ATG16L2_1_SG QT00005915), GAPDH (Hs_GAPDH_2_SG QT01192646) and β-actin (Hs_Actb_2_SG QT01680476). The relative quantities of target gene mRNA compared against two internal controls, GAPDH and β-actin RNA, were measured by following a $\Delta C_T$ method employing an amplification plot (fluorescence signal vs. cycle number). The difference ($\Delta C_T$) between the mean values in the triplicate samples of target gene and those of GAPDH and β-actin mRNAs were calculated by iQ5 Optical System Software version 2.0 (Bio-Rad) and the relative quantified value was expressed as $2^{-\Delta C_T}$.

### *Human autophagy PCR array*

The Human Autophagy RT² Profiler PCR arrays (SA Biosciences, PAHS-084A) were used according to the manufacturer's protocol. The detailed list of these key autophagy-related genes can be found on the manufacturer's website (http://www.sabiosciences.com/rt_pcr_product/HTML/PAHS-084A.html). Using real-time quantitative PCR, we reliably analyzed expression of a focused panel of genes related to autophagy. Relative gene expressions were calculated using the $2^{-\Delta\Delta C_t}$ method, in which $C_t$ indicates the fractional cycle number where the fluorescent signal reaches detection threshold. The "delta–delta" method uses the normalized $\Delta C_t$ value of each sample, calculated using a total of five endogenous control genes (*B2M*, *HPRT1*, *RPL13A*, *GAPDH* and *ACTB*). Fold change values are then presented as the average fold change $2^{(\text{average}^{\Delta\Delta C_t})}$ for genes in ConA-treated U87 glioblastoma cells relative to control U87 cells in both siScrambled- and siMT1-MMP-transfected cells. Detectable PCR products were obtained and defined as requiring <35 cycles. The resulting raw data were then analyzed using the PCR Array Data Analysis Template (http://www.sabiosciences.com/pcrarraydataanalysis.php). This integrated web-based software package automatically performs all $\Delta\Delta C_t$-based fold-change calculations from our uploaded raw threshold cycle data.

### *Transfection method and RNA interference*

Cells were transiently transfected with 20 nM siRNA against MT1-MMP (Hs_MMP14_6 HP validated siRNA; QIAGEN, SI03648841) or scrambled sequences (AllStar Negative Control siRNA; QIAGEN, 1027281) using Lipofectamine 2000 (Invitrogen, 11668). MT1-MMP-specific gene knockdown was evaluated by qRT-PCR as described above.

### *Gelatin zymography*

Gelatin zymography was used to assess the extracellular levels of proMMP-2 and MMP-2 activities. Briefly, an aliquot (20 µL) of the culture medium was subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in a gel containing 0.1 mg/mL gelatine (Sigma-Aldrich, G2625). The gels were then incubated in 2.5% Triton X-100 (Bioshop, TRX506.500) and rinsed in nanopure distilled water. Gels were further incubated at 37°C for 20 h in 20 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij-35, 50 mM Tris–HCl buffer, pH 7.6, and then stained with 0.1% Coomassie Brilliant blue R-250 (Bioshop, CBB250) and destained in 10% acetic acid, 30% methanol in water. Gelatinolytic activity was detected as unstained bands on a blue background.

### *Immunoblotting procedures*

The following electrophoresis reagents were used: SDS (Sigma-Aldrich, L3771), acrylamide (Bioshop, ACR001.1) and bis-acrylamide (Bioshop, BIS001.100). Proteins from control and treated cells were separated by SDS–PAGE. After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes (Millipore, IPVH00010) which were then blocked for 1 h at room temperature with 5% nonfat dry milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris–HCl, pH 7.5) containing 0.3% tris-buffered saline tween-20 (TBST) (TBST; Bioshop, TWN510-500). Membranes were further washed in TBST and incubated with the MT1-MMP (Millipore, AB6004) and GAPDH (Clone 6C5; Millipore, MAB374) primary antibodies (1/1000 dilution) in TBST

containing 3% BSA and 0.1% sodium azide (Sigma-Aldrich, S2002), followed by a 1 h incubation with horseradish peroxidase-conjugated donkey anti-rabbit (Jackson Immuno Research Laboratories, 711-035-152) or goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, 115-035-062) at 1/2500 dilutions in TBST containing 5% nonfat dry milk. Immunoreactive material was visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, RPN3004).

*Detection of AVOs and of GFL-LC3 puncta*

U87 cells were serum-starved in media containing the different conditions of transfections (siRNA, cDNA plasmid recombinant forms of MT1-MMP) or treatments with ConA (Sigma-Aldrich, L7647). Acridine Orange (0.5 ug/mL; Sigma-Aldrich, A6014) was added to each well, cells were incubated for 10 min at 37°C in the dark. Fluorescence was then examined by microscopy. Transient cell transfection with pEGFP-LC3 (generously provided by Dr Patrick Labonté, INRS-IAF, Qc), Wt-MT1-MMP, Δ-Cyto-MT1-MMP and ΔTM-MT1-MMP cDNA plasmids was performed using lipofectamine 2000 as described previously (Proulx-Bonneau, Guezguez et al. 2011; Proulx-Bonneau, Pratt et al. 2011).

*Statistical data analysis*

Data are representative of three or more independent experiments. Statistical significance was assessed using Student's unpaired *t*-test. Probability values of $<0.05$ were considered significant and an asterisk identifies such significance in the figures.

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## Conflict of interest statement

B.A. holds a Canada Research Chair in Molecular Oncology from the Canadian Institutes of Health Research (CIHR). R.R. holds a Canada Research Chair in Medicinal Chemistry from the Natural Sciences and Engineering Research Council of Canada (NSERC). J.P. is an NSERC awardee.

## Abbreviations

ATG12, autophagy-related protein 12; ATL16L1, autophagy-related protein 16-like 1; ATG3, autophagy-related protein 3; AVO, acidic vesicular organelle; BNIP3, BCL2/adenovirus E1B 19 kDa interacting protein 3; BSA, bovine serum albumin; ConA, concanavalin-A; COX-2, cyclooxygenase-2; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinases; GFP-LC3, green fluorescent protein - microtubule-associated protein light chain 3; MT1-MMP, membrane type-1 matrix metalloproteinase; NF-kB, nuclear factor-kappa B; PTEN, phosphatase and tensin homolog; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; SDS-PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; SHP, SH2-domain containing phosphatase; TBST, tris-buffered saline tween-20.

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