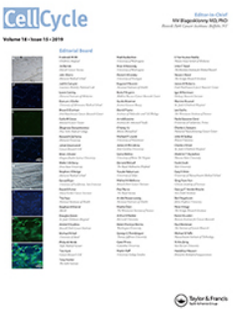

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REVIEW

'Patchiness' and basic cancer research: unravelling the proteases

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ABSTRACT

The recent developments in Cathepsin protease research have unveiled a number of key observations which are fundamental to further our understanding of normal cellular homeostasis and disease. By far, the most interesting and promising area of Cathepsin biology stems from how these proteins are linked to the fate of living cells through the phenomenon of Lysosomal Leakage and Lysosomal Membrane Permeabilisation. While extracellular Cathepsins are generally believed to be of central importance in tumour progression, through their ability to modulate the architecture of the Extracellular Matrix, intracellular Cathepsins have been established as being of extreme significance in mediating cell death through Apoptosis. With these two juxtaposed key research areas in mind, the focus of this review highlights recent advancements in how this fast-paced area of Cathepsin research has recently evolved in the context of their mechanistic regulation in cancer research.

Abbreviations : ECM, Extracellular Matrix; MMP, Matrix Metalloproteases; LL, Lysosomal Leakage; LMP, Lysosomal Membrane Permeabilisation; LMA, Lysosomorphic Agents; BC, Breast Cancer; ASM, Acid Sphingomyelinase; TNF-α, Tumor Necrosis Factor-alpha; LAMP, Lysosomal Associated membrane Protein; PCD, Programmed Cell Death; PDAC, Pancreatic Ductal Adenocarcinoma; ROS, Reactive Oxygen Species; aa, amino acids.

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Introduction

Tumor metastasis has been long known to be of central importance in malignancy and represents itself as a key step in tumor progression that may be amenable to therapeutic intervention in the fight against cancer. In line with neoplastic transformation of cells, the emergence of the Extracellular Matrix (ECM) has been given great significance over the last 20 years with a view to targeting it for therapeutic benefits based upon it promoting tumor cell proliferation, chemotherapeutic resistance, angiogenesis and providing the microenvironment essential for tumor survival and metastasis[1]. The basis of these efforts stemmed from observations that tumor cells possess enhanced proteolytic activity which permits them to breakdown ECM components and thus take on greater motility through the basal lamina during tumor metastasis [2].

Generally speaking, the extracellular proteins responsible for enhanced proteolytic activity of tumor cells include the Matrix Metallo- (MMPs)-[3] and additional serine-, threonine-, aspartic- and cysteine-proteases [4]. In the absence of any effective MMP-directed therapeutics having emerged [5], mainly due to their broad activity and toxic side effects [6], it is therefore not surprising that as an alternative approach, the Cathepsin proteases have recently developed into an intense area of study with a view to dissecting their regulatory mechanisms in cancer cell biology with therapeutic development as a foresight. While the superfamily of Cathepsin proteins has developed over a long time span, each member has been shown to possess distinct (and sometimes over-lapping) roles in their contribution to tumor progression based on their level and distribution of expression either at the subcellular level or at the extracellular level within tissues. In large, such studies have also given arise to a number of interesting research avenues that have focused on defining with greater clarity the substrate-specific protein sequences cleaved by well-characterized Cathepsins and Cathepsin-directed regulatory and effector signaling pathways. Consequently, areas of research that have flourished more recently include harnessing the

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potential of the Cathepsins in the design of novel cancer therapeutics based on Cathepsin-specific cleavage sites, with a view to harnessing their activity against tumor cells in conventional chemotherapy and more so against cells that exhibit chemotherapeutic resistance or metastasis.

As the role of intracellular Cathepsins in recent efforts have provided some very novel and exciting insights into firmly establishing their role in cell demise, the emergence of Lysosomal Leakage (LL) and Lysosomal Membrane Permeabilisation (LMP) has simultaneously gathered importance in the sense that this presents a key (and distinct) step in the initiation and execution of Cathepsin-dependent cell death. Initially, most of the limited evidence to support these beliefs were derived from the use of Lysosomorphic Agents (LMA), which destabilize the lysosomal membraine, resulting in the release of Cathepsin proteases into the cytoplasm and thus triggering cell death through caspase-dependent apoptosis (or Lysosomal Cell Death, LCD) [7]. However, such mechanisms have been verified and extended to offer greater biological significance in systems utilizing naturally-occurring apoptosis-inducing agents such as TNF, FAS, TRAIL and products of oxidative stress-all of which have offered a clearer insight into the biological, physiological and regulatory relationship between LL, LCD and the Cathepsins [8–12]. Moreover, such results have been arrived at with some interesting (and sometimes surprising) observations that have helped to drive this area of research into looking at what other key signaling cascades may also be connected to the Cathepsins before, during and after the initiation of LL in disease.

Based upon these collective findings as a backdrop, in which intracellular Cathepsins share a central importance in LCD while extracellular Cathepsins potentiate ECM remodeling and tumor metastasis, the regulation of Cathepsins may offer a more focused picture for improving the therapeutic development of these proteases further and which forms the core of this review article.

The Cathepsins and cancer

Over the years the central importance of the Cathepsins in disease have been intensely explored, particularly in the context of tumor proliferation, metastasis, invasion, ECM degradation, angiogenesis [13] and inflammation [14]. Consequently, it is with these perspectives in mind that the driving force behind Cathepsin research has broadly diversified with significant progress being made in certain areas of applied research, such as antibody-drug conjugates, diagnostic imaging and their use in targeted drug delivery [14,15]. More importantly, the focus of Cathepsin protease regulation has synergistically evolved to address their role in modulating the immune response and inflammatory cells (such as macrophages), both of which are central to maintaining the tumor microenvironment and the ECM during cancer and some inflammatory diseases (for an excellent review, see Kramer *et al.* [14]).

To date, the Cathepsin super-family of proteases constitutes a family of 15 lysosomal proteases which can be broadly classified into aspartic (D, E)-, serine (A, G)- and cysteine (B, C, F, H, K, L, O, S, V, Z/X, W)-proteases subtypes. Furthermore, such proteases can be subdivided based on their proteolytic activity into endo-peptidases (S, K, V, F, L) and both endo- and exo-peptidase (B, H, Z/X, C), of which Cathepsins C, B,H, K, O, L, Z/X are expressed broadly and which have been the most extensively studied to unveil general mechanism in substrate cleavage, lysosome research and cell death in disease [16] (see Table 1).

As seen from expression profiling studies, a number of Cathepsins have been shown to be highly expressed during tumor invasion and metastasis [32] (Table 2). For example, Cathepsin A was observed to be highly expressed in metastatic lesions of Malignant Melanoma [33], increased Cathepsin B protein expression was seen at the invasive edges of B16 Melanomas cancer cells [34], in patients with enhanced inter-pulmonary metastases [35] and Cathepsin D was seen to be over-expressed in metastatic Breast Cancer (BC) cells [36]. A number of studies have also supported the central role of Cathepsins in tumor invasion and metastasis using a variety of approaches. For example, overexpression of Cathepsin D in fibroblast cells resulted in increased fibroblast motility and invasion [37], Cathepsin Z/X upregulation was correlated with higher invasiveness *in vitro* [38], the importance of Cathepsin H in glioblastoma cell line invasion was demonstrated using Matrigel assays [39], Cathepsin K had enhancing effects on breast tumor epithelial

Table 1. Amino acid (aa) length and tissue-specific distribution of expression for the Cathepsins (Liver, Kidney, Thyroid Gland, Spleen, Placenta, Brain, Heart, Skeletal Muscle, Testis, Ovary, Macrophages, Cytotoxic T Lymphocyte, Osteoclasts, Epithelial cells of the GastroIntestine, Embryonic Respiratory and Urinary Tract and Foetuses, Lung, Lymph Nodes, Antigen Presenting Cells, Cornea, Thymus, Skin, Monocytes, Neutrophils, Melanoma, Plasma, Platelets, Intestine, Stomach, Erythrocytes, Prostate).

Cathepsin	(aa)	Expression	Reference
B	339	L,K,TG,Sp	Chan *et al.*[17]
C	463	Lu,Sp,K,P,CTL	Paris *et al.*[18]
F	484	B,H,SM,T,O,M	Wang *et al.*[19]
L	333	L,TG,K	Joseph *et al.*[20]
H	335	L,K,Sp	Fuchs *et al.*[21]
K	329	Os,M,EGI,ERUTF,Lu	Inaoka *et al.*[22]
O	321	L,K,P,O	Santamaria *et al.*[23]
S	331	Sp,LN,APC,H	Shi *et al.*[24]
V	334	C,T,Th	Bromme *et al.*[25]
W	376	Sp,LN,CTL	Linnever *et al.*[26]
Z/X	303	L,K,P,Lu	Nagler *et al.*[27]
A	480	B,S,P,PL,L	Galjart *et al.*[28]
G	225	S,Mo,N	Salvesen *et al.*[29]
D	412	Sp,K,L,Me,Pla,Plat	Faust *et al.*[30]
E	401	B,I,S,Er,LN,S,Sp,Lu	Azuma *et al.* [31]

Table 2. Expression levels of the Cathepsin proteins have been documented to cause Tumor Invasion (TI), Metastasis (M), Tumor Growth (TG), Extracellular Matrix Degradation (ECMD) or Angiogenesis (ANG) inMalignant Melanoma, Lung, Breast, Colon, GlioBlastoma, Hepatocarcinoma, Glioma, Ovarian, Squamous Cell Carcinoma, Basal Cell Carcinoma, Gastric, Prostate Cancer and Hepatocarcinoma .

Cathepsin	Cancer	Reference
B	TI/M	MM, L, B, C, GB, H[41]
H	TI/TM	GB[39]
L	TG/TI/ANG	PC[42,43]
K	TI/TM/ECMD	SCC, BC, G[44]
S	TI	C, GAS[45]
Z/X	TG/TI/TM	PC, HPC[46,47]
A	TI/TM	MM[48]
D	TI/TM/ANG	B, L, O [49]

cell development in Cathepsin K-positive fibroblast co-cultures [40] and Cathepsin S knockout and mutant Cathepsin S expression resulted in reduced invasiveness of pancreatic cancer [2].

Of importance has been Cathepsin B, the expression of which is elevated at the genomic, proteomic [50] and serum levels while often being linked to tumor progression in advanced stage cancer patients [51] such as in BC malignancy [52]. Herein, the underlying involvement of Cathepsin B has been verified by a number of approaches using overexpression, antisense [53], siRNA and shRNA studies [54] that have characterized and established the importance of Cathepsin B at various stages of cancer development. Moreover, as to be expected from a large family of proteases, some Cathepsins share some redundancy with regards to their modes of activity as seen with Cathepsins B and Z/X in cancer invasiveness [55]. As seen in overexpression studies, MCF7 cells underwent Epithelial-Mesenchymal Transition (EMT) while knockdown expression resulted in Mesenchymal-Epithelial Transition. Additionally, using such approaches, Cathepsin B has been further characterized in its role as promoting or reducing angiogenesis. Here, Cathepsin B knockout leukocytes showed reduced CD18 shedding and an accumulation in angiogenic vessel with reduced extravasal activity [56]. Collectively, such studies do indeed highlight the potential pleiotropic input of the Cathepsins in a variety of important processes in disease that extend from cellular transformation of cells to angiogenesis of tumor metastases and further work is indeed warranted in this area to define more clearly the key regulatory steps that give rise to Cathepsin-mediated activity.

Likewise, Cathepsin L research is also emerging as an interesting area of cancer research as Cathepsin L knockdown studies demonstrated abrogated tumor proliferation, growth and invasiveness in BC cells [57,58] while also rendering Glioma cells sensitive to radiotherapy treatment [58]. Interestingly, such silencing studies have also revealed the contribution that the Cathepsins can potentially make to chemoresistance, as seen in the case of Cathepsin L, in which silencing sensitized BC cells [59,60] and A549 lung cancer cells [61] to Paclitaxel and Cisplatin treatments. Such studies therefore suggest an important aspect of Cathepsin expression in connection with their contribution to conventional chemotherapeutic resistance while highlighting the importance of maintaining efforts on novel Cathepsin-specific therapeutic design.

In light of chemoresistance, the recent significance of Cathepsins expression in cancer stem cells have also gained momentum. As a developing area of interest, aberrantly expressed Cathepsins B and D transcripts and proteins were identified in Tongue Squamous Stem Cells using the techniques of Immunohistochemistry (IHC) staining and Nanostring/CISH mRNA analysis [62] and enhanced expression in liver metastases from

adenocarcinoma OCT4-positive cells [63]. In further developments, Cathepsin B activity was also found to be enriched in Glioblastoma stem cells from hypoxic niches using IHC and fluorogenic substrates in CD133-positive cells [64] indicating the possible importance of Cathepsin B as a potential marker in stem cell biology and cancer prognosis. Collectively, the potential involvement of the Cathepsins in cancer stem cell regulation appears to have significant and more research is therefore warranted in determining more precisely what mechanistic role they play in tumor signaling, progression and chemotherapeutic resistance in such systems.

Signalling networks and Cathepsin expression

Cathepsin transcriptional regulation

Based upon the observations that Cathepsin overexpression correlates strongly with tumor growth and progression, more recent efforts have therefore been directed at defining with greater clarity Cathepsin gene regulation and the contribution it may make to disease progression.

Consequently, the regulation of Cathepsin B was found to be modulated at the transcriptional level under INF-γ [65] or IL-6 cytokine stimulatory conditions [66] and transcription factors such as SP1 (-Cathepsin B) [67], SP3 (-Cathepsin L) [68] and Upstream Stimulatory Factors, USF (-Cathepsins B and L) [69,70] observed to be key regulators of Cathepsin gene expression. Similarly, using expression and knockdown studies of transcription factor FOXO3a in a gastric cancer model, Yu *et al.* (2016) demonstrated its ability to positively regulate the Cathepsin L promoter and protein expression, which had the effect of promoting EMT of cells by it reducing E-Cadherin protein levels [71]. More recently, developing efforts have also identified a number of other key transcription factors and the involvement of micro-RNAs in regulating a number of Cathepsin transcripts. For example, transcription factor FOXM1 was found to bind and activate the Cathepsin B promoter in gastric cancer cells [72] and as reported by Luan *et al.* (2018) [73], Cathepsins B and L were overexpressed in an MZF1 transcription factor-dependent manner, in BC cells [73]. Finally, Cathepsins B and L protein secretion was also dependent on active transcription factors Ets1, SP1, NF-κB during EMT in primary melanoma invasiveness [74]. While such findings clearly demonstrate a link between signal transduction pathways and the regulation of Cathepsin genes at the transcriptional level, greater clarification is needed on whether such signaling cascades (and intermediates) may affect the activity of the Cathepsins at the protein level.

More recently, Cathepsin L transcriptional repression has been demonstrated to be regulated by mir-152 in gastrointestinal tumors and the expression of which induced apoptosis and inhibited proliferation, migration, and invasion of gastrointestinal stromal cells [75]. Similarly, Cathepsin A transcription was targeted by expressed mir106b-5p causing suppression of Colorectal Carcinoma (CRC) cell migration and invasion [76]. Interestingly, mir200c overexpression reduced Cathepsin L expression in lung A549 cells, resulting in enhanced susceptibility to Paclitaxel-mediated cell death and EMT suppression [77]. While, such recent studies link Cathepsin transcriptional expression to an alternative mode of regulation through the miRNAs, more importantly, such studies also highlight the relative importance of miRNA as a potential therapeutic in overcoming Cathepsin-mediated chemoresistance and Cathepsin-mediated EMT regulation [77]. As seen previously, mir152 has been linked to regulating Wnt-mediated EMT inhibition [78] and mir106b-5p with the regulation of key signaling intermediates such as GSK3B, VEGFA and PTK2 in colon and cervical cancers [79] and in both of which, Cathepsins A and L are seen to play a vital regulatory role. Thus, it is conceivable that these miRNAs could be regulating these Cathepsins and other key signaling pathway intermediates simultaneously, or exclusively in a time-dependent and coordinated manner. In similar studies, mir200C was found to inhibit EMT (or lung cancer cell invasion *via* HMGB1 signaling) [80] while also being able to reverse EMT [81]-both of which may have possible regulatory inputs from Cathepsin L expression *via* this microRNA.

As an emerging area of interest, further exploration of Cathepsin gene regulation by microRNAs

that also modulate other key oncogenes or tumour suppressors and which may act in concert (or crosstalk) with the Cathepsin (at the protein or transcriptional level) is therefore much needed.

As an additional feature of Cathepsin gene transcription, alternative exon splicing [82] and exon skipping [83] can give rise to protein forms translated from additional downstream ATG [84] start codons that do not enter the secretory pathway (due to them lacking the required secretory signal) and can therefore enter the nucleus.

Post translational activation of Cathepsins

As the Cathepsins superfamily members vary in their polypeptide lengths, general tissue expression or distribution and their intracellular (or extracellular) localization in normal or disease states (Tables 1 and 2), for simplicity we will aim to focus on the most characterized of these to highlight the general pathways responsible for protein maturation after translation. For example, Cathepsin B is synthesized in the Rough Endoplasmic Reticulum (RER) as a 339 amino acid (aa) protein containing a 17 aa signal peptide [85]. Following insertion into the RER lumen, the signal peptide is removed and the 43/46 KDa inactive pro-Cathepsin B precursor is glycosylated and transported to the Golgi where it is further glycosylated with phosphor-mannose residues. Following this, it binds the Mannose-6-Phosphate Receptor (M6PR) allowing transportation from the trans-Golgi to the late endosome (where the acidic environment permits the intermolecular pro-domain removal [86] and then on to the lysosomal compartment. It is through this key endosomal cleavage (and regulatory) step, that Cathepsin D (an aspartic protease) can also activate Cathepsin B [87], as can Cathepsin G, urokinase-type Plasminogen Activator (uPAR) and tissue-type plasminogen activator and elastasase proteins [88,89]. Further cleavage of Cathepsin B generates a double chain form consisting of a 25 KDa heavy and 5 KDa light chain [90]. Interestingly, Cathepsins can also undergo transport from the Golgi to the lysosome independently of the M6PR. For example, Sortilin was found to transport Cathepsin D and Cathepsin H in this manner [91] and in a recent study by Boonen *et al.* (2016), the protein SEZ6L2 was discovered to be a novel substrate for Cathepsin D and which could transport Cathepsin D to the endosomes (from the Golgi and cell surface) in Hela and neuroblastoma cells [92] (see Figure 1). Once activated (in the late endosomes), Cathepsin activity can also be negatively (and tightly) regulated by naturally-occurring endogenous inhibitors, of which there are four main groups named the Stefins, Cystatins, Kininogens and non-inhibitory homologues [16]. Structurally, Cathepsin B is bilobal [93] and can act as an endo-peptidase at neutral pH or an exo-peptidase (with carboxypeptidase activity) at acidic pH [94]. Like Cathepsin B, Cathepsin H can also be trans-activated (by Cathepsin L) and for both of which strong supporting data has been derived from structural studies [95,96].

Secretion of Cathepsins

In contrast to normal cells, during malignancy the Cathepsins have been observed to localize at the cell periphery and in exocytic vessicles after the traversing the trans-Golgi [97]. More recently, the respective importance of cell surface V-ATPase and extracellular acidification have been reported for the secretion of active or non-active Cathepsin B [98] and Cathepsin D [99] in metastatic BC cells [98]. Such studies offer greater clarity in defining broad regulatory steps involved in active Cathepsin secretion during cancer progression and identify what other protein intermediates regulate this key step in Cathepsin localization. Collectively, these observations offer support to the belief that in cancer cells, lysosomes behave differently by relocating from a perinuclear location to the cell periphery allowing their contents to be readily localized to the ECM [100]. However, more studies are required to ascertain mechanistically which proteins are directly involved in anterograde and retrograde trafficking of the active Cathepsins, particularly in cells that contain dysfunctional lysosomes or cells derived from different tumor compartments or stages of tumor development.

Lysosomal leakage as a key regulatory mechanism for cell death

While lysosomes were discovered over 50 years ago as subcellular organelles responsible for the

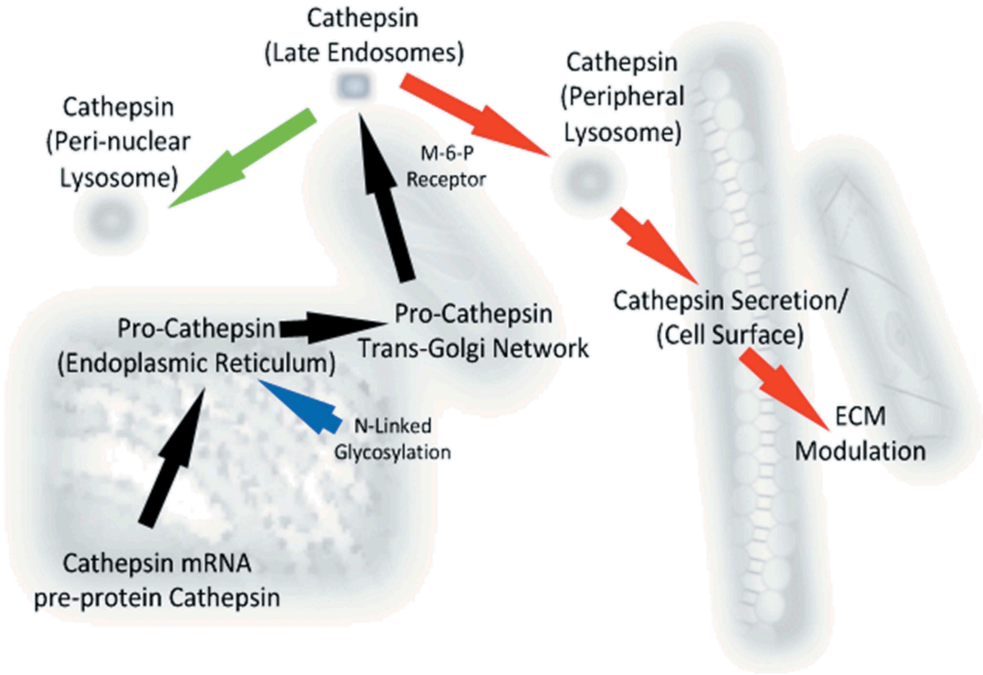

Figure 1. Schematic showing the synthesis, trafficking and maturation of Cathepsin proteins in normal cells (green arrows) and cancer cells (red arrows). Pro Cathepsin mRNAs are translated, inserted into the Endoplasmic Reticulum where they are glycosylated and transported to the Golgi network. Upon further glycosylation, they are transported in a Mannose-6-Phosphate (M-6-P)-dependent manner to the late Endomes after which they can enter the Perinuclear Lysosome or the Peripheral Lysosome and be secreted to modulate the Extracellular Matrix (ECM, red arrows).

degradation of extracellular materials (taken up by cells through phagocytosis) or the intracellular degradation of complex molecules (such as DNA, proteins, lipids and carbohydrates), they were also described as "suicide bags" that can cause tissue autolysis upon rupture [101,102]. From these initial observations, compelling evidence has accumulated to support their distinct and unique role in regulating apoptosis through the activation of the Caspases, thus giving them center stage in the areas of auto-inflammatory disease and cancer research [103]. However, in the field of Cathepsin research, we are presented with a paradox in the sense that while secreted extracellular Cathepsins can give rise to metastatic disease [32,104], deregulated intracellular Cathepsin expression (derived from LL) is of equal importance in determining the apoptotic fate of tumor cells [10,105]. Consequently, the lysosomal localized portion of Cathepsins may offer an axis of regulation that can be exploited for therapeutic purposes and has therefore generated great interest over the years [104,106].

Defining the involvement of the lysosome at the molecular level and its involvement in cell fate with greater clarity is an area that has indeed developed more recently through the understanding of LL which occurs as a consequence of LMP. In comparison to normal cells, cancer cells are believed to have larger, more fragile lysosomes [107] and which are more sensitive to disruption by agents that result in LMP. Agents which can trigger this change have given rise to a wealth of knowledge in how the lysosome is linked to apoptosis in cancer cells (See Figure 2).

Firstly, lysosomes are sensitized to Reactive Oxygen Species (ROS), which are produced at higher levels in cancer cells [108] due to a range of stimuli such as drugs, heavy metals [109] and conditions such as ischaemia and inflammation [109].

Secondly, the study of LMAs have yielded some interesting insights into how LMP can be selectively

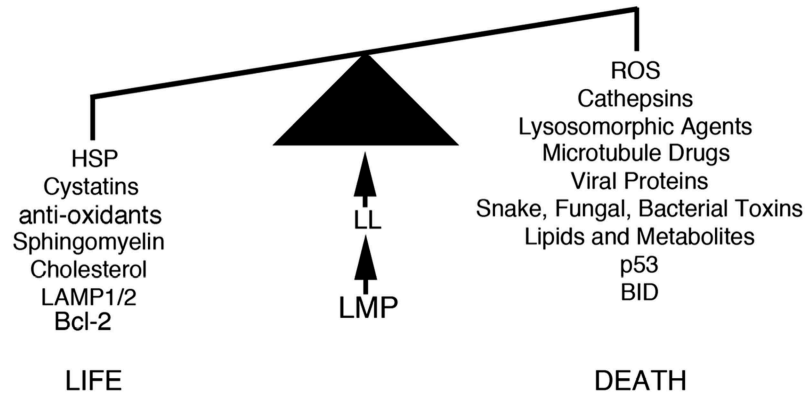

Figure 2. Lysosomal Membrane Permeability (LMP) and Lysosomal Leakage (LL) determine cell survival based on the cellular expression levels of Heat Shock Proteins (HSP), Cystatins, anti-oxidants, Sphingomyelin, Cholesterol, LAMP1/2 and Bcl-2. Cell death can also be induced by the lysosome, based upon cellular levels of Reactive Oxidative Species (ROS), Cathepsin, Lysosomorphic Agents, Lysosomal Leakage, Microtubule Disrupting drugs, Viral Proteins, Toxins, Lipids and Metabolites, protein p53 and protein BID (see text for more details).

induced in cancer cells [110]. Good examples of these include amines with hydrophilic side chains (for example, imidazole [102], Ciprofloxacin [105], Sphingosine [111] and Siramesine [112,113]). More recently, a Riccardin D-derivative was also seen to trigger LMP and Cathepsin B release in Prostate Cancer cell apoptosis. Interestingly, it also caused Cathepsin B to relocate from the lysosomes to the nucleus where it was observed to potentiate DNA damage by suppressing BRCA1 activity, thus highlighting a novel role for nuclear Cathepsin B activity [114].

Thirdly, Sphingosine accumulation due to the activation of lysosomal Acid Sphingomyelinase (ASM) and acid Ceramide production can also activate LMP. While other signaling intermediates which trigger LMP (including phospholipase-A2) have been long known about [115], more recently the treatment of rat hepatocytes with Tumor Necrosis Factor-alpha (TNF-α) was also seen to induce LMP [116]. In support of this, inhibition of Sphingokinase 1 (SPK1, which converts sphingosine to sphingosine-1-phosphate, SP1) also induced LMP, with the loss of S1P giving rise to abnormal lysosomes [117]. The finding that Sphingokinase 1 is also a Cathepsin B substrate offers additional support to the alternative manner in which Cathepsin B can exert its death-inducing activity [118,119].

Fourthly, p53 is believed to induce LMP in Myeloid leukemia cells [120] and TNF-α treated Fibrosarcoma cells [121]. Here, the dependency of LMP activation was influenced by the presence of phospho-ser15-p53 at the lysosomal membraine [121] and the involvement of any other isoforms of p53 in LMP activation are yet to be defined [122]. As an unexaustive list, other agents are also capable of inducing LMP such as microtubule targeting drugs [123,124], viral entry proteins [125] and cobra snake venom toxins [126].

Conversely, we must not disregard the role of proteins and intermediates which can also stabilize and safeguard the lysosome against the above damaging agents [127], such as Heat Shock Proteins (HSP, HSP70 and HSP90), Mcl-1/BclX_L, anti-oxidants (such as Vitamins C and E, Super Oxide Dismutase, Glutathione Peroxidases and Catalases), Cholesterols, Sphingomyelin and Lysosomal Associated Membraine Proteins (LAMP) 1 and 2 [127]. Generally speaking, deregulated expression of these products may give rise to resistance in Cathepsin-mediated death under circumstances where Cathepsin expression levels are detected as being relatively high enough to trigger the onset of LMP (under normal conditions). Good evidence to support the effect of Cathepsin expression and LMP onset also comes from treating rat hepatocytes with TNF-α (or Sphingosine) in cells lacking Cathepsin B and in which LMP was observed to be ablated [116]. Similarly, Cathepsin D was also shown to induce LMP upon TNF-R1 internalization in a Caspase-8 and −7 dependent manner and which activated ASM [128]. Mechanistically, Cathepsin-induced

LMP is believed to occur upon the intra-lysosomal degradation of highly-glycosylated LAMPs, which form a glycocalyx shield on the inner side of the lysosomal membraine [129,130].

Lysosomal Membrane Permeabilisation regulation by the Cathepsins

Lysosomal-mediated cell death can take on a number of forms depending on the types of agents that adversely affect the integrity of the lysosome [110,131].

Upon robust LMP induction, the morphological features of cell death are not witnessed and uncontrolled necrosis occurs instead. Conversely, in the presence of limited LMP, apoptosis [111,132] or caspase-independent death can be activated [133] and readily visualized. Under the former of these circumstances, the Cystatins fail to attenuate cell death unless they are over-expressed suggesting the central importance of Cathepsin expression levels (in relation to endogenous Cystatins) in death pathway induction. Nevertheless, LMP-induced apoptosis is activated through Mitochondrial Outer Membrane Permeabilization (or MOMP) and Cytochrome C release [134], which can be brought about by Cathepsin-mediated BID cleavage (and its activation to pro-apoptotic tBID) or by inhibiting the cleavage of anti-apoptotic Bcl_2, BCl-xl and Mcl-1 proteins [135–137]. Moreover, the caspases can be activated directly upon their cleavage by Cathepsins or by cleavage of their inhibitors such as the XIAP proteins [137] (Figure 3).

LMP can also cause cell death with no caspase or little caspase activation (even in the instance of HSP70 depletion [138]), upon hypochlorous acid [139] and Siramesine treatment of cells [112,113]. Here, inhibition of activated caspases did not seem to reduce cell death suggesting that the Cathepsins can take on a role as alternative executors of cell death [16] and that other uncharacterized signaling mechanisms may be involved at the molecular level of Cathepsin regulation and activity.

Additionally, it is also worth noting the unique relationship that appears to be developing based on the recent observations that Cathepsin expression contributes to cell death [118,119] and how silencing of Cathepsins can sensitise cells to conventional chemotherapy and DNA-damaging agents [59–61]. While these present potentially conflicting roles for the Cathepsins, it clearly highlights the importance of precisely delineating the contribution of the Cathepsins to other key life-death signaling pathways (or *vice versa*) in future studies.

Signal transduction intermediates and Cathepsin regulation

As the field of Cathepsin research has developed at a phenomenal pace over the last 5 years, it therefore comes as little surprise that the regulation of these proteins at the transcriptional and the post-translational level are being viewed in a broader context with regards to how they may crosstalk or interact with other signaling cascades (or intermediates). For example, Cathepsin D was found to cleave Bcl-2 causing the sensitization of BC cells to TRAIL-mediated death, thus linking Cathepsin D activity with cleavage of negative (or pro-survival) regulators of PCD [140]. Moreover, Fritsch *et al.* (2016) confirmed BID cleavage by Cathepsin D (resulting in Caspase-9 activation) and identified a novel substrate for Cathepsin D (using proteomics) as HSP90 [141]. Analysis of HSP90-Cathepsin D cleavage-null mutants, partially prevented apoptosis of U937 and Jurkat cells highlighting a central role for Cathepsin D in HSP90 level regulation in LL, LMP and apoptosis. Meanwhile, Burton *et al.* (2017) linked nuclear Cathepsin L with CUX1 proteolytic processing and thus linked Cathepsin L with EMT (and MET) progression in BC and PC cells [142]. Interestingly, in this study subcellular Cathepsin L was also observed to translocate from the nucleus to the cytoplasm, highlighting a novel protein trafficking-function for Cathepsin L. Kim *et al.* (2018) showed that Cathepsin S could bind BRCA1 and facilitate it's ubiquitination and breakdown, thus suppressing DNA repair in BC development, while knocked-down expression of Cathepsin S stabilized BRCA1 levels, thus collectively linking Cathepsin S to BRCA1 signaling transduction [143]. As a novel substrate, Bian *et al.* (2015) found Cathepsin B to colocalise and target $p27^{kip}$ for degradation in CRC cells, thus linking it to cell cycle modulation [144].

Bannoud *et al.* (2018) found that Cathepsin D can bind the M6PR and are co-transported in an estradiol

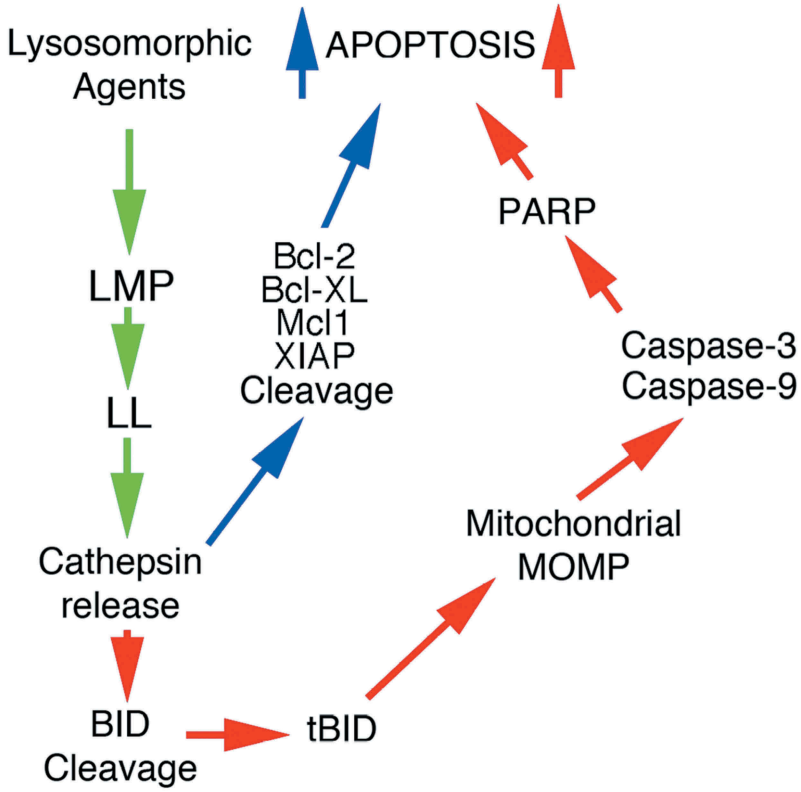

Figure 3. Schematic showing the events leading to Cathepsin protein release (green arrows) following the induction of Lysosomal Membrane Permeabilisation (LMP) and Lysosomal Leakage (LL) which result in apoptosis, through Mitochondrial Outer Membrane Permeabilization (MOMP) and Poly (ADP-ribose) polymerase (PARP) activation (red arrows) *via* Caspases-3 and −9. The blue arrows highlight enhanced apoptosis through the activity of Cathepsin-mediated cleavage of anti-apoptosis proteins (see text for more details).

stimulatory manner through MCF-7 cells and which linked Cathepsin D trafficking with estradiol-dependent EMT progression (and possible chemoresistence) [145]. Additionally, chemokines CXCL-9 and −10 were seen to upregulate Cathepsin B expression and secretion in BC cells *via* CXCR3 signaling [146], while the tumour suppressor Protilin was identified as a novel substrate for Cathepsin Z/X, the cleavage of which reduced its binding to Clathrin, thus linking Cathepsin Z/X activity and regulation of Clathrin-dependent endocytosis [147]. Recently, Shao *et al.* (2018) showed that NEDD4 was needed for EGFR-dependent lung cancer cell migration and EGF-induced Cathepsin B secretion, as seen from a lack of Cathepsin B secretion in analysing a ligase-dead mutant of NEDD4 and in NEDD4 knockdown experiments [148]. Finally, BAG2 was observed to process pro-Cathepsin B auto-activation and facilitated the secretion of pro-Cathepsin B to the cell surface, thereby increasing its metastatic potential in triple-negative breast cancer cells and thus linking BAG2 oncogenic signaling with Cathepsin B activation and tumor invasiveness [149].

Collectively, all these findings suggest the importance of Cathepsin regulation during cancer progression and which may be more complex than once thought. However, novel mechanisms are being unveiled through which the Cathepsins may impose their proteolytic activity on other signaling intermediates and thus modulate (or be modulated by) other key signaling and protein trafficking cascades that have already been firmly implicated in disease or cell survival. When taken together, all of the above findings contribute greatly to our clinical understanding of the Cathepsins and allows researchers to view them in a broader context either in model systems or in disease.

Reflective and future directions

The last 3 years in Cathepsin research and cancer biology have presented themselves as being

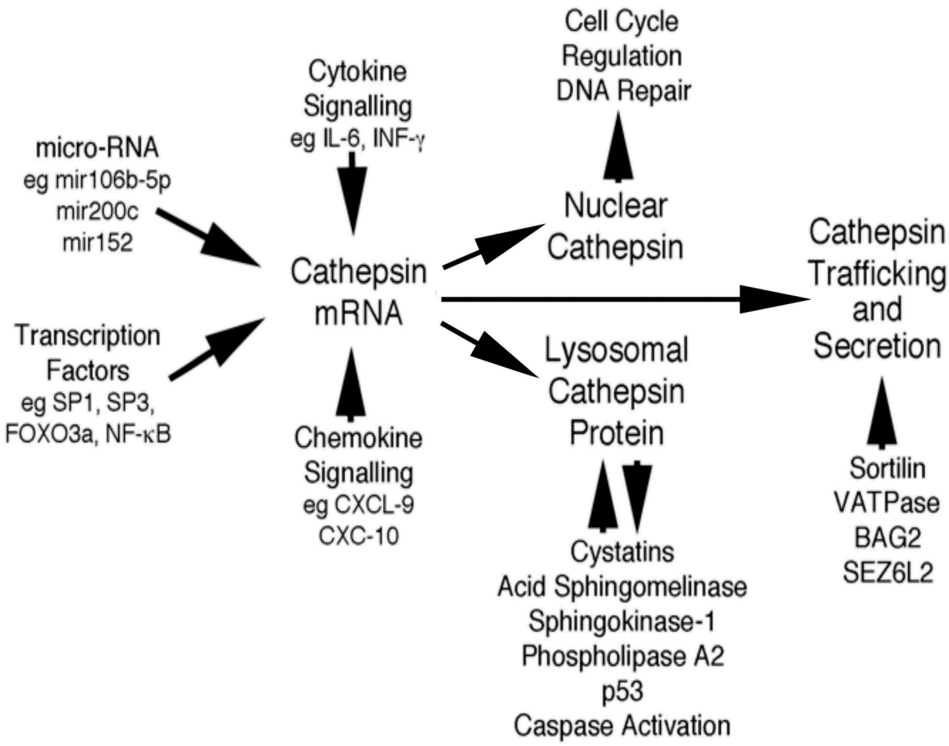

Figure 4. Schematic highlighting the regulatory inter-relationships between transcription factors, micro-RNAs (mir), Cytokine/Chemokine signalling, Cathepsin inhibitors and signalling intermediates, DNA repair and cell cycle regulators and trafficking proteins with Cathepsin gene regulation, protein trafficking and secretion (see text for details).

incredibly diverse and hugely productive. In reflection, and based on the literature published, there are many new and exciting areas of basic research that are emerging or coming into fruition and which appear to be moving basic research towards a translational phase at a phenomenal pace (Figure 4). In reflection, what started off as a cytoplasmic organelle responsible for the general disposal and degradation of subcellular matter is slowly emerging as an organelle that has many other key regulatory functions in normal cellular homeostasis. As reviewed herein, the peripheral lysosome can indeed contribute to apoptosis while simultaneously being the source of secreted Cathepsin proteins and thus offers a clearer understanding of how the Cathepsins can be more realistically targeted during therapeutic design.

Taken with the identification of novel substrates (or binding partners) for the Cathepsins and cross-talk between key signaling, transcriptional or protein trafficking pathways, the future of research in this area holds great promise in unravelling with even greater clarity the regulation of the Cathepsins while identifying more potential therapeutic targets.

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Ethical standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human

experimentation and with the Helsinki Declaration of 1975, as revised in 2008. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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