

**Effects of impaired proteostasis on
tissue physical properties in a model of
bone-like tissue formation**

Thesis submitted by

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Abstract

Cellular function and viability and thus tissue health and integrity depend on the maintenance of protein homeostasis (proteostasis) by controlled protein degradation. Prolonged dysregulation of proteostasis is associated with age-related diseases such as neurodegenerative disorders, diabetes, and cancer. However, as chronic imbalances rather than acute loss of proteostasis mediate the pathogenesis of these diseases, research models that allow for the study of the complex effects of drugs on tissue properties are almost completely lacking. The question of how impaired proteostasis affects tissues rather than single cells is also of relevance for proteostasis-targeting drugs, which are increasingly used in cancer therapies.

In this project I aimed to determine the functional effects of impaired proteostatic fine-tuning by applying a combination of materials science characterisation techniques to a cell-derived, *in vitro* model of bone-like tissue formation in which I pharmacologically perturbed protein degradation. I demonstrate that low-level inhibition of VCP/p97 and the proteasome, two major components of the protein degradation machinery, have remarkably different effects on the bone-like material that human bone-marrow derived mesenchymal stromal cells (hMSC) form *in vitro*. Specifically, whilst proteasome inhibition mildly enhances tissue formation, Raman spectroscopic, atomic force microscopy-based indentation, and electron microscopy imaging reveal that VCP/p97 inhibition induces the formation of bone-like tissue that is softer, contains less protein, appears to have more crystalline mineral, and may involve aberrant micro- and ultra-structural tissue organisation. These observations

contrast with findings from conventional osteogenic assays that fail to identify any effect on mineralisation. Taken together, these data suggest that mild proteostatic impairment in hMSC alters the bone-like material they form in ways that could explain some pathologies associated with VCP/p97-related diseases. They also demonstrate the value of quantitative materials science approaches for tackling long-standing questions in biology and medicine and could form the basis for preclinical drug testing platforms to develop therapies for diseases stemming from perturbed proteostasis or for cancer therapies targeting protein degradation. The findings may also have important implications for the field of tissue engineering, as the manufacture of cell-derived biomaterial scaffolds may need to consider proteostasis to effectively replicate native tissues.

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Declaration of originality

I hereby declare that all the work included in this thesis is my own unless otherwise stated and has been referenced accordingly.

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Disclosures

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An engineered quantifiable in vitro model for analysing the effect of proteostasis-targeting drugs on tissue physical properties

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Aspects of the work presented in this thesis, in particular the human bone marrow mesenchymal stromal cell expansion, have contributed to other research papers on which doctoral candidate Sandra Loaiza is an author (Ferreira et al., 2018a, Ferreira et al., 2018b, Taheem et al., 2018).

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List of abbreviations

ABBREVIATION	FULL NAME
AFM	Atomic force microscopy
ALP	Alkaline phosphatase
ALPL	Alkaline phosphatase
ALS	Lateral sclerosis
APC	Allophycocyanin
ARS	Alizarin red S stain
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
Atg12	Autophagy related 12
Atg5	Autophagy related 5
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma 2
BGLAP	Bone gamma-carboxyglutamate protein
BIP	Immunoglobulin heavy-chain-binding protein
BM	Bone marrow
BMP2	Bone morphogenic protein 2
BMP2	Bone morphogenetic protein 2
BRC	Biomedical Research Centre
BS1	Binding site 1 motif
BSE	Backscattered electrons
BSP	Bone sialoprotein
Btz	Bortezomib
Ca	Calcium
CBF-alpha-1	Core-binding factor subunit alpha-1
CD	Cluster of differentiation
Cdc48	Cell division cycle
cDNA	Complementary deoxyribonucleic acid
CHOP	Transcription factor C/EBP homologous protein
Cl	Chlorine
CO ₂	Carbon dioxide
Col1 α 1	Collagen type 1 alpha 1
CRL	Cullin RING ligase
DBeq	<i>N</i> 2, <i>N</i> ⁴ , <u>d</u> ibenzylquinazoline-2-4-diamine
DDC-SEM	Density-dependent colour scanning electron microscopy
DDIT3	DNA damage inducible transcript 3

DDIT3	DNA damage inducible transcript 3
dH ₂ O	Distilled Water
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNAJC3	DNA J heat shock protein family (Hsp40) member C3
Dsk2	Shuttling factor
DTT	Dithiothreitol
ECM	Extracellular matrix protein
EDEM	ER degradation-enhancing α -mannosidase-like protein
EDTA	Ethylenediaminetetraacetic acid
eIF2	Elongation initiation factor 2
ER	Endoplasmic Reticulum
ERAD	Endoplasmic reticulum-associated degradation
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
GADD34	Growth arrest and DNA damage-inducible 34
GAPDH	Glyceraldehyde-3-phosphatase dehydrogenase
GMP	Good manufacturing practice
GPI	Glycosylphosphatidylinositol
GRP94	Glucose regulated protein 94
GvHD	Graft versus Host Disease
H ₂ O	Water
HAS	Human albumin solution
HCl	Hydrochloric acid
HIF1 α	Hypoxia inducible factor-1 alpha
HLA-DR	Human Leukocyte Antigen – DR isotype
hMSC	Human mesenchymal stromal cells
HSC	Haematopoietic stem cells
Hsp	Heat shock protein
HSPA5	Heat shock protein family A member 5
HTA	Human Tissue Authority
Hz	Hertz
IBMPFD	Inclusion body myopathy associated with paget's disease of the bone and fronto-temporal dementia
IBSP	Integrin binding sialoprotein
IC ₅₀	Inhibitory concentration
ICHTB	Imperial College Healthcare Tissue Bank
IFN- γ	Interferon gamma
IHCNT	Imperial College Healthcare NHS Trust
IRE1	Inositol-requiring enzyme 1
ISCT	International Society for Cellular Therapy
JNK	c-Jun N-terminal kinase
KO	Knockout

LC3	Protein light chain 3
LMP	Proteasome subunit
MEM	Minimum essential medium
MgF ₂	Magnesium fluoride
MM	Multiple myeloma
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stromal cell
mTORC1	Mammalian target of rapamycin complex 1
NaOH	Sodium hydroxide
NFE2L1	Nuclear factor, erythroid-2-like-1
NFE2L1	Nuclear factor, erythroid 2 like 1
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NH ₄ OH	Ammonium hydroxide
NHS	National Health Service
NIHR	National Institute for Health Research
Npl4	Nuclear pore localisation protein 4
NZF	Npl4 zinc finger
OB	Osteoblast
OC	Osteoclast
ON	Osteonectin
OPM-2	Multiple myeloma cell line
OST	Oligosaccharyltransferase
Osx	Osterix
PBS	Phosphate buffered saline
PBU	PLAA-family ubiquitin binding
PCR	Polymerase chain reaction
PDI	Protein disulphide isomerases
PE	R-phycoerythrin
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PhD	Doctor of Philosophy
PI	Proteosome inhibition
PI3K	Phosphatidylinositol 3-kinase
PL	Platelet lysate
PO ₄ ³⁻	Phosphate
PP1	Protein phosphatase 1
PP2	Protein phosphatase 2
PPlases	Peptidylprolyl- <i>cis/trans</i> -isomerases
PPP1R15A	Protein phosphatase 1 regulatory subunit 15A
PPP1R15A	Protein phosphatase 1 regulatory subunit 15A
PUB	Peptide N-glycosidase/ubiquitin-associated
PUL	PLAA, Ufd3 and Lub1
PVDF	Polyvinylidene difluoride

Rad23	Shuttling factor
REC	Research ethics committee
RNA	Ribonucleic acid
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
RUNX2	Runt-related transcription factor 2
S1P	Site-1protease
S2P	Site-2 protease
Saos-2	Human osteosarcoma cell line
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel
SE	Secondary electron
SEM	Scanning electron microscopy
Shp1	Suppressor of high copy PP1
SPARC	Secreted protein acidic and cysteine rich
SPR	Signal recognition particle
STR	Short tandem repeat
TBST	Tris-buffered saline, tween20
TCF11	Transcription factor 11
TEM	Transmission electron microscopy
TNC	Total nucleated cell count
TNF α	Tumour necrosis factor alpha
TRAP	Translocon-associated-protein
TXNIP	Thioredoxin interacting protein
UBA	Ubiquitin-associated
UBD	Ubiquitin-binding domain
UBX	Ubiquitin regulatory X
UBX-L	Ubiquitin regulatory X-like
Udf1	Ubiquitin fusion degradation 1
UPR	Unfolded protein response
UPS	Ubiquitin proteasome system
VCP	Valosin-containing protein
VIM	VCP-interaction motif
XBP1	Transcription factor spliced X-box protein 1
α -MEM	Minimum essential medium alpha

1 Introduction

1.1 Protein homeostasis

Maintenance of cellular protein homeostasis or proteostasis, is essential for tissue integrity and has been linked to longevity (Kaushik and Cuervo, 2015, Labbadia and Morimoto, 2015, Perez et al., 2009, Schneider and Bertolotti, 2015a). Perturbed proteostasis contributes to the pathogenesis of numerous age-related diseases ranging from neurodegenerative disorders such as Alzheimer's disease or Parkinson's disease to diabetes and cancer (Clarke et al., 2014, Hetz and Mollereau, 2014, Mukherjee et al., 2015).

An intricate network of cellular systems constantly monitors and fine-tunes the intracellular proteome (Balchin et al., 2016, Hetz and Mollereau, 2014). Proteostasis requires a dynamic coordination of protein synthesis, protein folding of newly synthesized proteins, conformational maintenance, quality control, and controlled degradation of dysfunctional, damaged, or no longer needed proteins (Figure 1) (Klaips et al., 2018). The ubiquitin proteasome system (UPS) is the principal cellular pathway for controlled protein degradation and achieves two main objectives, the degradation of protein 'waste' and the recycling of amino acids for both energy generation and synthesis of new proteins. Autophagy is an alternative protein degradation pathway responsible for recycling damaged organelles and macromolecules.

Cell

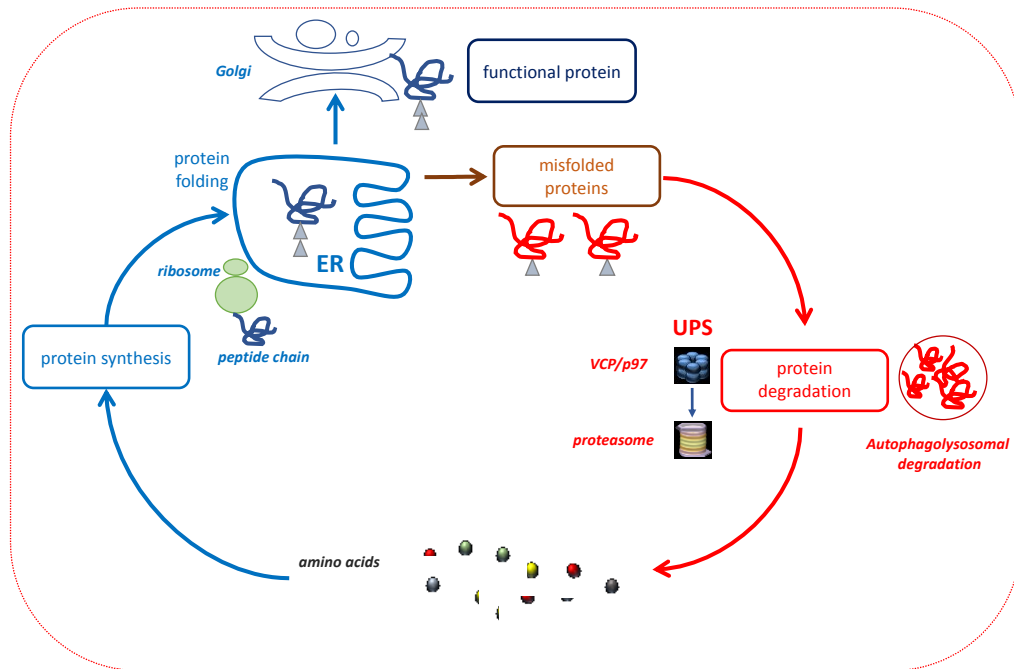


Figure 1. Proteostasis network. Schematic depicting the proteostasis system within cells that includes protein synthesis at the ribosome, folding in the endoplasmic reticulum (ER), packaging of correctly assembled proteins in the Golgi apparatus, accumulation of misfolded proteins and degradation of misfolded proteins into amino acids by the ubiquitin proteasome system (UPS) or removal of protein aggregates from damaged and dysfunctional proteins by the autophagy-lysosomal pathway.

Several components of the UPS are the targets of approved and investigational drugs for cancer therapies (Backer et al., 2011, Forsythe et al., 2018, Shen et al., 2013). Proteins that are secreted or membrane-bound make up approximately one third of cellular proteins and require complex post-translational modifications. These proteins enter the endoplasmic reticulum (ER) largely co-translationally where they fold and assemble. Proteins that are properly assembled pass the quality control mechanisms and advance from the ER to the Golgi apparatus and cell surface. Proteins that fail quality control mechanisms accumulate as 'misfolded/unfolded' proteins and cause a condition called ER stress. ER stress causes the activation of an adaptive cellular mechanism, the unfolded protein response (UPR) that aims to restore secretory homeostasis. If the UPR fails to restore homeostasis, accumulation of defective proteins causes overwhelming ER stress leading to programmed cell death that usually occurs in the form of apoptosis, a tightly regulated sequence of cellular events that results in the disposal of a cell. ER stress and a dysregulated UPR have been implicated in a myriad of diseases (Clarke et al., 2014, Hetz and Mollereau, 2014, Mukherjee et al., 2015). Furthermore, the UPR has other important functions playing a critical role in cell differentiation, a fully functional humoral immune response (Gass et al., 2004, Iwakoshi et al., 2003a, Iwakoshi et al., 2003b, Reimold et al., 2001, Zhang et al., 2005) and the proper formation of bone (Jang et al., 2012, Murakami et al., 2009, Tohmonda et al., 2011, Wei et al., 2008, Zhang et al., 2019).

In this study, I investigated the effects of impaired proteostasis on tissue formation, composition and physical properties and function in an *in vitro* model of bone-like tissue.

1.2 Protein folding in the ER

The majority of newly synthesised proteins need to undergo post-translational modifications of the nascent polypeptide to attain their final configuration making the protein 'fit' for secretion and able to function (Anelli and Sitia, 2008). Protein modifications which lead to correct 'folding' in the ER include elimination of disulphide bond formation between cysteines, glycosylation, covalent modifications, hydroxylation, carboxylation, acetylation, methylation, amidation, deamidation, phosphorylation, ADP-ribosylation, oligomeric assembly and ubiquitination, for which the tubular ER network provides optimal conditions (Dobson, 2004, Ellgaard et al., 2016). Most of these modifications and various quality control procedures, take place in the ER where rigorous control mechanisms are in place to stop secretion of non-functional proteins with only properly assembled proteins evolving from the ER to the cell surface. These quality control mechanisms regulate whether a protein travels from the ER along the secretory pathway, is retained for further modifications, or is returned to the cytosol for ER-associated degradation (ERAD) (Adams et al., 2019, Anelli and Sitia, 2008, Malhotra and Kaufman, 2007).

Newly synthesised proteins that are still bound to the ribosome in an unfolded state are targeted and translocated across or inserted into the membrane of the ER through a cylindrical transmembrane channel, the protein translocon. The translocon comprises the heterotrimeric Sec61 channel, which is composed of an α , β , and γ subunit and several additional proteins and complexes that are permanently or transiently associated with the Sec61 complex (Lang et al., 2017, Song et al., 2000).

Following targeting to the ER, precursor polypeptides with amino-terminal signal peptides associate with the Sec61 complex via their targeting peptide activating opening of the Sec61 channel. Some precursor polypeptides require help from other additional proteins and complexes for Sec61 channel opening such as the membrane protein complex 'translocon-associated-protein' (TRAP) complex or immunoglobulin heavy-chain-binding protein (BiP, also known as GRP78 and HSP5A) (Sommer et al., 2013). BiP (Grp78), an ATP and Ca^{2+} -dependent heat shock protein (Hsp) from the Hsp70 family, binds to their substrates through hydrophobic domains in the ER luminal side (Adams et al., 2019). Although BiP is the most abundant classical chaperone in the protein control system in the ER, there are other ER-associated classical co-chaperones such as Hsp40 and Hsp90 (Lang et al., 2017).

Membrane translocation of polypeptides is regularly accompanied by protein modifications such as glycosylation by the enzyme oligosaccharyltransferase (OST), glycosylphosphatidylinositol (GPI) anchor attachment by GPI transaminase, or removal of signal peptides by signal peptides enzymes (Kinoshita, 2014, Lang et al., 2017). Once the polypeptide emerges from the Sec61 translocon, concurrent folding and assembly of the polypeptide commences. This process is assisted and controlled by chaperones and folding enzymes (Lemus and Goder, 2014). The main components of this chaperone network are BiP that acts in conjunction with its Hsp-40 co-chaperones (ERjs or ERdj) and nucleotide exchange factors (NEFs) Sil1 and Grp170 (Ellgaard et al., 2016). Moreover, folding can involve additional chaperones, such as glycoprotein-specific calnexin (CNX) and calreticulin (CRT) that play a key role in glycosylation by adding N-linked glycans to the newly synthesised protein which positively affects glycoprotein folding capacity, and folding catalysts such as

protein disulphide isomerases (PDIs) and peptidylprolyl-*cis/trans*-isomerases (PPIases). Eventually native polypeptides that have passed these quality control mechanisms are passed on from the ER along the secretory pathway (Lang et al., 2017).

Conditions such as oxidative stress, hypoxia, starvation, inflammation as well as high secretory demand or insults to the folding process, can overwhelm the ER's folding capacity, resulting in accumulation of unfolded or misfolded proteins in the lumen of the ER which are non-functional and prone to aggregation. This condition, referred to as ER stress, triggers the activation of the unfolded protein response (UPR) (Kondo et al., 2012, Walter and Ron, 2011).

1.3 Unfolded protein response

The UPR is a homeostatic mechanism by which cells regulate levels of misfolded proteins in the ER thereby maintaining a productive ER-protein folding environment. The UPR involves a series of intracellular signal transduction pathways that together aim to restore secretory homeostasis by transiently attenuating protein translation, increasing the capacity of protein trafficking through the ER, increasing production of molecular chaperones involved in protein folding and transport, and enhancing protein degradation pathways including ERAD (Figure 2) (Walter and Ron, 2011).

ER stress activates three ER-transmembrane UPR sensors – protein kinase RNA-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6).

The first signalling pathway of the UPR is mediated by PERK. PERK is an ER-resident transmembrane protein with a cytosolic serine/threonine kinase domain encoded by the *EIF2AK3* gene. Under non-stress conditions, heat shock protein 90 (Hsp90) and BiP bind to the cytoplasmic and ER luminal domains of PERK respectively to stop activation (Marcu et al., 2002). Detection of misfolded proteins by the luminal domain of PERK, triggers dimerization and *trans*-autophosphorylation of itself and elongation initiation factor 2 (eIF2 α). PERK phosphorylates the α subunit of eIF2 α to inhibit translation while inducing activating transcription factor 4 (ATF4) to promote ER adaptation. ATF4 has multiple target genes of which GADD34 (growth arrest and DNA damage-inducible 34) and CHOP (transcription factor C/EBP homologous protein) are particularly relevant for the regulation of the UPR. GADD34 is a regulatory subunit of the protein phosphatase 1 (PP1) that promotes eIF2 α dephosphorylation. As eIF2 α phosphorylation attenuates mRNA translation, fewer

misfolded proteins accumulate in the ER thereby promoting cell survival (Figure 2) (Walter and Ron, 2011). CHOP is a transcription factor that regulates genes encoding factors involved in apoptosis. CHOP is upregulated downstream of ATF4 in response to ER stress causing downregulation of the anti-apoptotic mitochondrial protein B-cell lymphoma 2 (Bcl-2). Thus, the PERK pathway protects the cell at moderate levels of stress but signals to cell death under severe ER stress. Programmed cell death usually occurs in the form of apoptosis, a tightly regulated sequence of cellular events.

The second signalling pathway of the UPR is mediated by IRE1. IRE1 increases the transcription of genes encoding chaperones when it senses stress in the ER (Schneider and Bertolotti, 2015b). Mammals have two IRE1 genes, *IRE1A* (also known as *ERN1*) which encodes IRE1 α , and *IREB* (also known as *ERN2*) which encodes IRE1 β . Like PERK, IRE1 α is a transmembrane protein with a cytosolic serine/kinase domain. Under low stress conditions, Hsp90 and Hsp72 bind the IRE1 α cytosolic domain to maintain stability, while BiP binds the luminal domain of IRE1 α to prevent dimerization (Wang and Kaufman, 2014). During ER stress, unfolded and misfolded proteins bind BiP, thereby releasing IRE1 α for oligomerization, auto-phosphorylation and activation of its kinase activities. IRE1 activates the transcription factor spliced X-box protein 1 (XBP1s) to induce genes that facilitate protein folding and removal of unfolded proteins by ERAD (Walter and Ron, 2011). IRE1 α cleaves Xbp1 mRNA to produce a translational shift generating a transcriptionally active form (*Xbp1s*) that enters the nucleus to regulate target genes. To accelerate the response, Xbp1u (unspliced) mRNA localizes to the ER membrane to facilitate IRE1 α interaction.

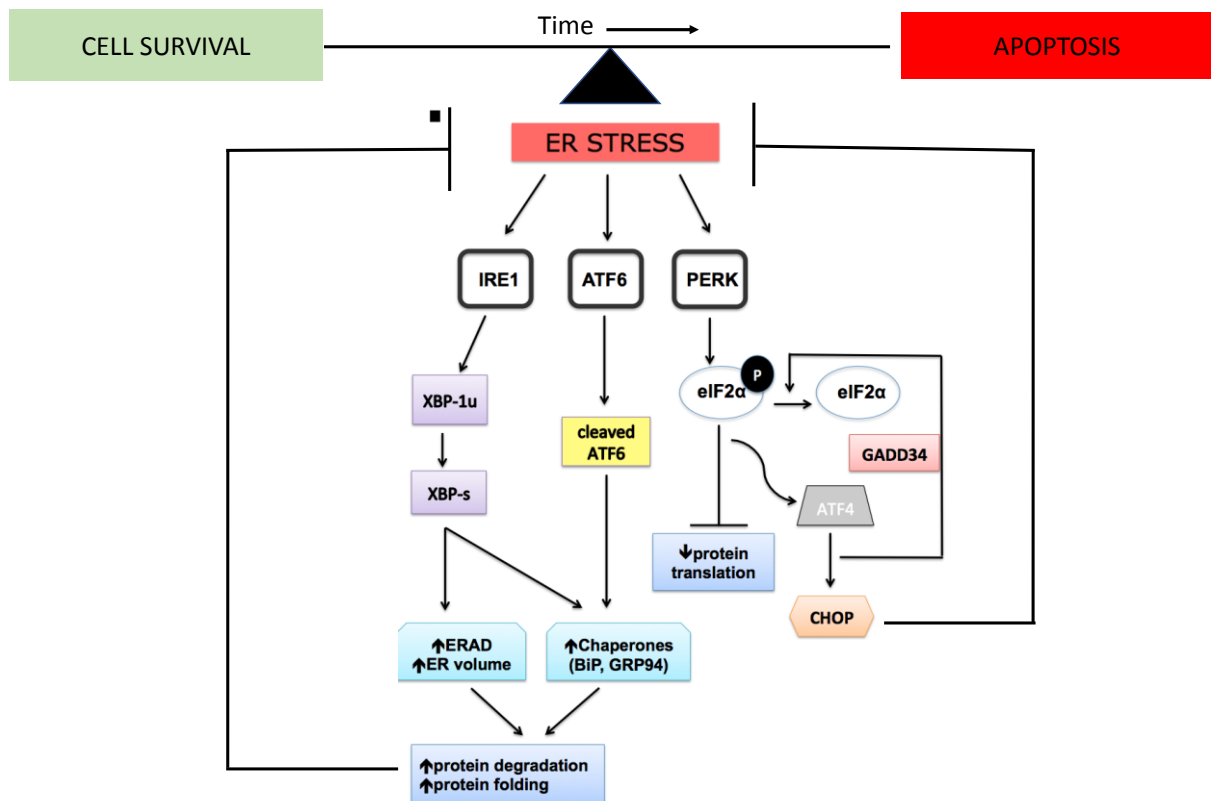


Figure 2. The UPR signaling network. A simplified diagram of the main components of the UPR. The UPR attempts to restore ER homeostasis through the activation of IRE1, ATF6 and PERK signaling pathways in response to ER stress. IRE1 activation results in the splicing of XBP-1, which induces the transcription of molecular chaperones, such as BiP and GRP94 (glucose-regulated protein 94), expands the ER and stimulates protein degradation via ER-associated degradation (ERAD). Activation and cleavage of ATF6 also leads to the induction of chaperones. PERK phosphorylates eIF2 α to inhibit protein translation. PERK also regulates several transcription factors including ATF4 and CHOP. This results in up-regulation of GADD34, which promotes de-phosphorylation of eIF2 α , thereby reversing inhibition of protein translation. In conjunction, the 3 pathways work as feedback loops that aim to alleviate ER stress. If the cell continues to be exposed to ER stress over time and the UPR is unable to restore secretory homeostasis, the cell enters apoptosis.

In this manner, genes that are regulated by IRE-XBP1 enhance protein folding, protein trafficking and ERAD, thereby resolving protein misfolding, and expression of XBP1 inhibits CHOP expression, thereby promoting cell survival (Figure 2) (Wang and Kaufman, 2014). Similar to the PERK pathway, if IRE signalling is not attenuated because of severe stress, chronic IRE activation signals apoptosis.

The third signalling pathway of the UPR is mediated by ATF6. ATF6 is a transcription factor that is synthesized as an ER-resident transmembrane with a large ER-luminal domain encoded by several genes. When unfolded proteins accumulate, it is packaged into vesicles in the ER and is delivered to the Golgi apparatus (Schindler and Schekman, 2009). In the Golgi, it meets two proteases, S1P and S2P (site-1 and site-2 protease) that in sequence remove the luminal domain and the transmembrane anchor respectively. The N-terminal cytosolic domain which is now free, moves into the nucleus to activate UPR target genes such as BiP and glucose regulated protein 94 (GRP94), a chaperone of the Hsp90 family (Figure 2). The exact mechanism by which ATF6 responds to ER stress is yet to be fully understood. ATF6 associates with BiP and the release of BiP under stress conditions is thought to contribute to its activation (Walter and Ron, 2011).

In summary, the UPR is an essential adaptive mechanism that exerts both protective and adverse effects on cell survival upon ER stress. This mechanism is designed to restore secretory homeostasis, however if stress signals persist over time, the mechanism signals apoptosis (Figure 2).

Moreover, the UPR has been demonstrated to play a crucial role in regulating cell differentiation, maturation and function of several tissue types. The transcription factor XBP1 is preferentially expressed in several foetal tissues, and mouse embryos

deficient in XBP1 die *in utero* (Clauss et al., 1993, Reimold et al., 2001). Late B cell development into antibody-secreting plasma cells has been shown to require XBP1 (Gass et al., 2004, Reimold et al., 2001). Studies with XBP1 deficient chimeric mice revealed that the animals had very low number of plasma cells which failed to mount an adequate humoral immune response to antigen *in vivo* (Reimold et al., 2001). XBP1 together with ATF4 have also been shown to participate in cell differentiation mechanisms in other tissues such as mammary epithelial cells (Tsuchiya et al., 2017). Recent studies have elucidated the function of the UPR in stem cells (Sigurdsson and Miharada, 2018, Yang et al., 2016). The UPR has been shown to regulate self-renewal capabilities of haematopoietic stem cells (HSCs) and gene expression analysis revealed that PERK is predominantly activated in HSCs compared to downstream progenitor populations (Yang et al., 2016). Bone progenitor osteoblasts derived from mesenchymal stromal cells have been demonstrated to depend on activation of the UPR for their differentiation, proliferation and function (Murakami et al., 2009, Tohmonda et al., 2011, Wei et al., 2008).

Furthermore, the importance of the UPR is also emphasised by studies that have demonstrated that both ER stress and UPR activation are involved in the pathology of cancer, inflammation, degenerative diseases, metabolic disease and immune disease (Wang and Kaufman, 2014, Yang et al., 2016).

1.4 ER-associated protein degradation

The ER is capable of recognising terminally misfolded, proteins without interfering with the functioning of the ER. When these proteins continue to be generated, they accumulate and aggregate. In such circumstances, the proteins have to be disposed of in an orderly way in order to alleviate ER stress. Although some misfolded proteins are transported to and degraded by autophagy in the lysosome (Hong et al., 1996), the bulk of protein degradation is executed by the UPS in a process referred to as ERAD (Wu and Rapoport, 2018). The UPS recognises proteins that have been earmarked for degradation by the addition of polyubiquitin chains that serve as a signal for proteasomal degradation thereby regulating multiple cellular functions and determining cell fate (Hershko and Ciechanover, 1998, Meyer, 2012, Moran-Crusio et al., 2012, Widjaja et al., 2017).

The close link between ERAD and quality control in the ER has been demonstrated by several studies showing that inhibition of the proteasome leads to the accumulation of misfolded proteins and secretory incapacity (Anelli and Sitia, 2008, Cenci et al., 2008).

1.4.1 Autophagic protein degradation

Macroautophagy (thereafter referred to as autophagy) is a tightly regulated process of bulk degradation and recycling of cytoplasmic organelles or cytosolic cellular components such as misfolded proteins. In this essential mechanism, a cell reacts to several stressful conditions such as hypoxia, reactive oxygen species, DNA

damage and protein aggregates by clearing aggregated and misfolded proteins, macromolecules or damaged organelles. Autophagy can promote cell survival or commit cells to non-apoptotic cell death depending on the type and duration of stress signals (Debnath et al., 2005).

In autophagy, cytoplasmic components of a cell are sequestered within double membrane vesicles, called autophagosomes, and delivered to the lysosome for degradation (Debnath et al., 2005). The initial stages of membrane formation require a protein complex that contains phosphatidylinositol 3-kinase (PI3K), p150 myristylated protein, and Beclin-1. The membrane extends around its target until the edges fuse to form the autophagosome (Levine and Klionsky, 2004). Extension of the membrane is mediated by a complex containing autophagy related 5 (Atg5) and autophagy related 12 (Atg12), and microtubule-associated protein light chain 3 (LC3). LC3 is essential for autophagosome conversion and is used as a marker for autophagy induction. The autophagosome then fuses with a lysosome developing a one-membrane vesicle called autolysosome. In the autolysosome, the target is degraded by lysosomal hydrolases allowing cells to eliminate damaged or harmful components, thereby maintaining nutrient and energy homeostasis (Kroemer and Jaattela, 2005, Parzych et al., 2015b). Thus, key functions of autophagy are quality control but also provision of recycled metabolites. Autophagy is also involved in a variety of biological functions like development, cellular differentiation, defence against pathogens and nutritional starvation (Ravanan et al., 2017).

1.4.2 ER-associated degradation (ERAD)

Protein quality control mechanisms in the ER selectively target terminally misfolded proteins to ERAD. The first step in ERAD is distinguishing terminally misfolded proteins from proteins that are still undergoing maturation. Although the exact mechanisms for this process are not fully understood, mannose trimming is thought to be involved. The most important enzymes involved in ERAD, are members of the ER degradation-enhancing α -mannosidase (EDEMs) protein family. Another group of proteins involved in ERAD are Derlins, which are induced by the mammalian ER stress response. Overexpression or knockdown of members of the Derlin family leads to enhanced and decreased degradation of misfolded proteins respectively (Oda et al., 2006, Olivari et al., 2005).

Once terminally misfolded proteins are identified and selected for ERAD, they undergo partial unfolding and reduction, a process in which both BiP and PDI are involved. Next, the target protein is exported from the ER lumen to the cytosolic side of the ER via the ER translocon membrane channel Sec61, a channel also used for translocation of newly synthesised polypeptides in the ER and earmarked for proteosomal degradation by polyubiquitination. Polyubiquitination acts as a signal for binding of the VCP/p97 complex that delivers the target protein and delivers it to the proteasome for degradation. In summary, this quality control process can be divided into four steps: substrate recognition, retro-translocation, substrate polyubiquitination and proteosomal degradation (Figure 3) (Dupzyk and Tsai, 2016).

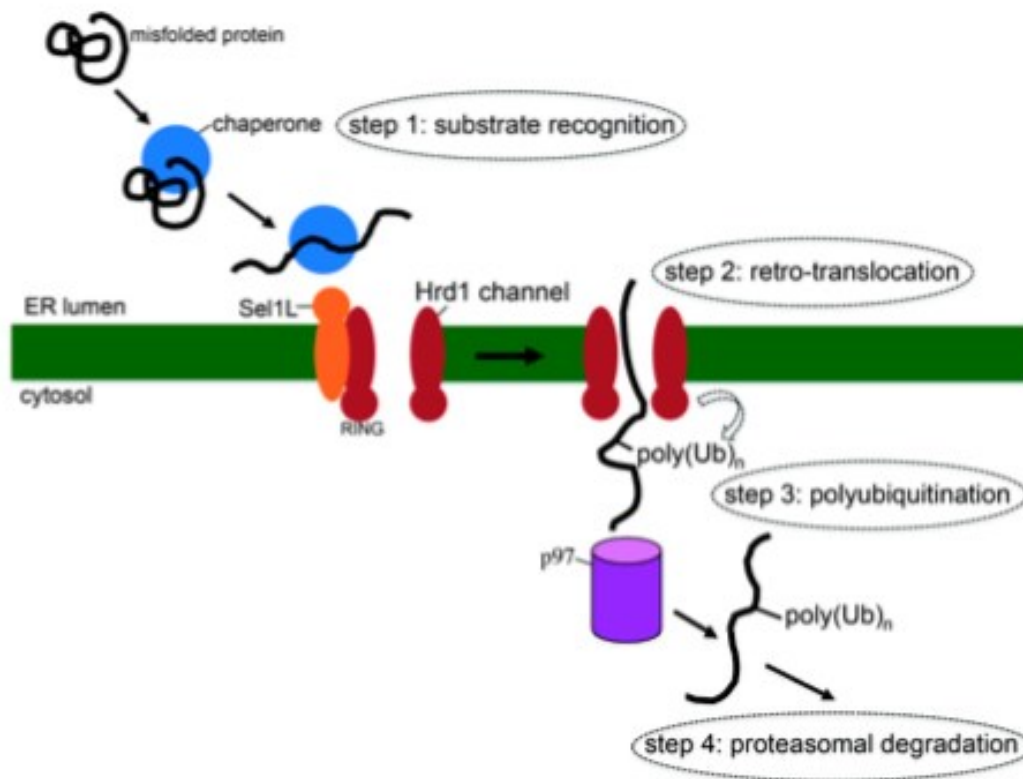


Figure 3. ER-associated degradation (ERAD). A misfolded protein (substrate) is recognized by ER chaperones (step 1) and is retro-translocated through an ER translocon membrane channel (step 2). The protein is then conjugated with chains of ubiquitin (Ub) resulting in polyubiquitination on the cytosolic side of the ER membrane (step 3). The VCP/p97 complex associates with the retro-translocation complex, recognizes the polyubiquitin chain and extracts the ubiquitinated protein to the cytosol. Polyubiquitin chains then direct the protein to the proteasome for degradation (step 4).

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1.4.3 Ubiquitination

Protein degradation through the UPS begins with the polyubiquitination of target proteins by a three-enzyme cascade involving the E1 ubiquitin-activating enzyme, an E2 conjugating enzyme and an E3 ligase that functions in the attachment of the ubiquitin to the substrate (Meyer, 2012, Zhi Guang et al., 2019). Ubiquitination is a post-translational modification that occurs in the cytosol and nucleoplasm of eukaryotic cells. Ubiquitin an 8 kDa polypeptide, is activated by its ATP-dependent C-terminal coupling to the thiol group of a specific cysteine of the E1 enzyme. Subsequently, ubiquitin is transferred to a specific cysteine of an E2 enzyme and finally to the substrate, usually the ϵ -amino group of internal lysines, which is presented to the E2-linked ubiquitin by an E3 ubiquitin ligase (Lemus and Goder, 2014). The E3 ligases with a function in ERAD are Doa10p and Hrd1p that are the core components of the Doa10p complex and the Hrd1p complex consisting of several membrane integrated proteins and associated components in the ER lumen and the cytosol. Doa10p and Hrd1p are multi-spanning ER membrane proteins and belong to the family of the RING (Really Interesting New Gene) domain in the cytosolic C-terminus which catalyses the transfer of ubiquitin from the E2 to the substrate (Deshaies and Joazeiro, 2009). The E2 enzymes with function in ERAD are Ubc6p and Ubc7p for Doa10p, and Ubc7p and Ubc1p for Hrd1p (Hirsch et al., 2009). The location of misfolded domain(s) on the ERAD substrate is a determining factor for which ERAD complex is used for their degradation. Membrane proteins with a cytosolic domain are usually targeted to the Doa10-complex, a pathway called ERAD-C (C for cytosolic).

Membrane proteins with a misfolded luminal domain are usually targeted to the Hrd1-complex, a pathway called ERAD-L (L for luminal). This same pathway degrades soluble proteins of the ER lumen. Proteins with a misfolded region in the transmembrane domain(s) are also targeted to the Hrd1-complex. This pathway is called ERAD-M (M for membrane) (Lemus and Goder, 2014). The exact mechanism of how individual proteins in the ER are first recognised is not fully understood but is likely to involve exposure of hydrophobic domains and binding of chaperones. The selected substrate is then targeted to the E3 membrane-bound ligating complex where they undergo ubiquitination on their protein domain exposed in the cytosol during or after retro-translocation.

1.4.4 VCP/p97

The valosin-containing-protein (VCP), also known as p97 or Cdc48 (Cell Division Cycle), is a AAA+ cytosolic ATPase that uses energy derived from ATP hydrolysis to remodel client proteins and engages in multiple cellular processes. It makes up 1% of the total cellular protein pool and is one of the most conserved proteins in eukaryotes highlighting its importance in cell homeostasis (Stolz et al., 2011, Wang et al., 2009).

The protein is mainly localised in the cytosol, but a considerable proportion is associated with membranes of various organelles such as the ER and the Golgi apparatus. A small quantity of VCP/p97 is also found in the nucleus (Madeo et al., 1998). VCP/p97 oversees critical steps in ubiquitin-dependent protein control and intracellular signalling pathways (Meyer, 2012). As previously mentioned, VCP/p97 plays a central role in the UPS by extracting ubiquitinated proteins and delivering

them to the proteasome for degradation (Meyer, 2012, Verma et al., 2011, Ye et al., 2017). However, VCP/p97 has also been linked to proteasome-independent handling of protein aggregates, autophagy, endosomal sorting, regulating protein degradation at the outer mitochondrial membrane and a role in chromatin-associated processes (Bastola et al., 2016, Deshaies, 2014, Shah and Beverly, 2015). Hence, VCP/p97 has a dual role in protecting cells from protein stress and ensuring genome stability during proliferation (Meyer, 2012).

1.4.4.1 VCP/p97 structure

VCP/p97 has a barrel shape with a homohexameric configuration, comprising of two ATPase domains (D1 and D2) and a globular N-terminal domain that interacts with several cofactors (Figure 4 a) (Tang et al., 2017). Both the D1 and D2 domains bind to ATP. However, the D2 domain has been shown to be primarily involved in the VCP/p97-ATPase activity, because mutations that abrogate ATP binding or hydrolysis result in dominant-negative variants that bind but cannot release substrates (DeLaBarre and Brunger, 2005, Pye et al., 2006). Moreover, the N-terminal domain is the main region for substrate and co-factor binding (Banerjee et al., 2016). It can stabilize unfolded proteins, may regulate ATP hydrolysis and even couple substrate and adaptor binding to ATP hydrolysis (DeLaBarre and Brunger, 2005, Pye et al., 2006).

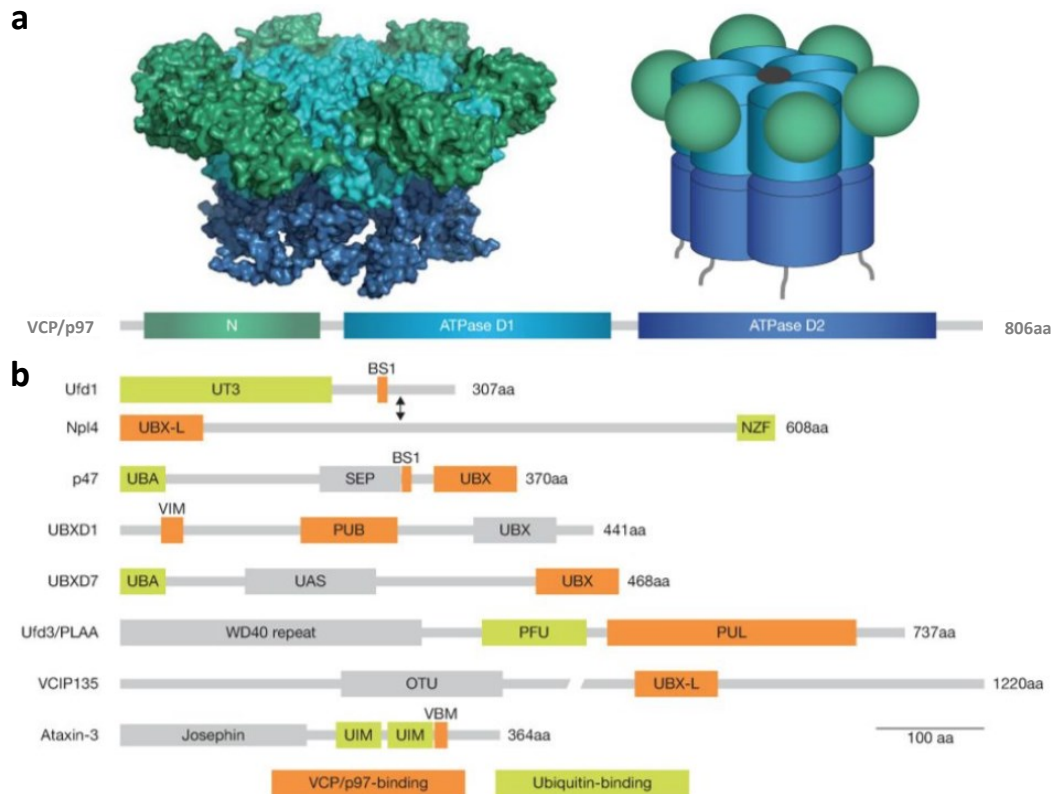


Figure 4. VCP/p97 structure and its cofactors. (a) VCP/p97 consists of a globular N-terminal domain (green), two AAA ATPase domains D1 (cyan) and D2 (blue), and a C-terminal tail (grey). D1 and D2 form two stacked hexameric rings. The N-terminal domain is placed on the outside of the D1 ring. ATP hydrolysis in D2 is understood to be the main driving force to induce rearrangements in VCP/p97 that may be transduced to target substrates to promote their structural re-modelling. **(b)** Domain structure of key VCP/p97 cofactors and adapters. UBX or UBX-like (UBX-L) or short motifs including the binding site 1 motif (BS1), the VCP-interaction motif (VIM) or the VCP-binding motif (VBM), interact with the N-domain. PUB (peptide N-glycosidase/ubiquitin-associated) or PUL (PLAA, Ufd3 and Lub1) domains bind to the C-terminal tail. Ubiquitin-binding domains, such as the ubiquitin-associated (UBA), the Npl4 zinc finger (NZF) or the PLAA-family ubiquitin binding (PFU) domains are shown.

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1.4.4.2 ***VCP/p97 function***

The cellular function of VCP/p97 is dependent on several regulatory cofactors. Interaction of VCP/p97 with client proteins is mediated by a range of ubiquitin cofactors and adapters which recognise both VCP/p97 and the ubiquitin chains in the client protein (Figure 4 b). VCP/p97 binds to the substrate (client protein) via a ubiquitin-adaptor containing a ubiquitin-binding domain (UBD) and a VCP/p97-binding UBX (ubiquitin regulatory X) or UBX-like (UBX-L) domain. VCP/p97 adapters can also link VCP/p97 to the E3 ligases that ubiquitylate the client proteins (Deshaies, 2014, Saffert et al., 2017). The largest family of cofactors are proteins containing a UBX or UBX-like domain that interact with the N-domain of VCP/p97 (Figure 4 b). Other domains such as PUB (peptide N-glycosidase/ubiquitin-associated) and PUL (PLAA, Ufd3 and Lub1) and shorter interactive motifs, have also been identified to bind to the C-terminal or the N-domain respectively (Figure 4 b). These interactions are essential for VCP/p97 activity and functional diversity and define the destination a protein takes within a pathway, for example whether a substrate is processed or degraded (Stolz et al., 2011).

1.4.4.3 ***Role of VCP/p97 in protein degradation***

The role of VCP/p97 in ubiquitin-dependent processes and ER-associated degradation (ERAD) is well established. VCP/p97 complexes bind to ubiquitinated substrates and uses the energy of ATP hydrolysis to structurally remodel or unfold its client protein to enable downstream processes (Meyer, 2012). These processes include extracting ubiquitylated targeted protein from cellular components,

segregating protein from binding partners or generating initial unfolded stretches to facilitate degradation by the proteasome (Meyer, 2012, Song et al., 2015). Reducing the functionality of VCP/p97 either by siRNA in cells or immunodepletion in *in vitro* protein degradation assays suppresses the degradation of polyubiquitinated proteins (Song et al., 2015).

Thus, VCP/p97 is a key member of the ubiquitin-proteasome system, playing a central role in ER-associated degradation (ERAD) and thus essential for cellular homeostasis. In ERAD, VCP/p97-adaptor complexes, ubiquitin fusion degradation 1 (Ufd1) and nuclear pore localisation protein 4 (Npl4) (Figure 4 b), bind polyubiquitinated substrates and retro-translocate them from the ER lumen to the cytoplasm to enable degradation by the proteasome (Meyer et al., 2002, Ye et al., 2003).

Song and colleagues (Song et al., 2015) demonstrated that VCP/p97 can unfold proteins conjugated with polyubiquitin chains. This activity is consistent with the function of VCP/p97 in proteasomal degradation as the proteasome has a narrow opening that only allows the passage of unfolded polypeptides. The unfolding activity of VCP/p97 is thought to facilitate proteasomal degradation in a number of ways; unfolding misfolded proteins facilitating their retro-translocation from the ER lumen to the cytosol, synergy in substrate unfolding with the proteasome's regulatory complex of highly folded ubiquitinated proteins, and binding to the proteasome to form an additional hexameric ATPase ring to alleviate stress due to deficient unfolding activity in response to proteasomal impairment.

Several studies have revealed an additional function of VCP/p97 in coordinating ubiquitination and downstream ubiquitin editing events by binding E3 ubiquitin ligases including cullin RING ligases (CRLs), directly or indirectly through adaptors

(Alexandru et al., 2008, Bandau et al., 2012, den Besten et al., 2012). Conversely, VCP/p97 binds deubiquitinating enzymes which are suggested to remove ubiquitin to assist substrate recycling and act as positive regulators of protein degradation. Some studies suggest that VCP/p97 passes substrates over to the shuttling factors Rad23 and Dsk2 to deliver them to the proteasome (Richly et al., 2005), others suggest that VCP/p97 is required for substrate remodelling at the proteasome rather than delivery (Verma et al., 2011).

In summary, VCP/p97 is a key player in proteasomal degradation, essential for cellular homeostasis in response to demands of different cellular activities. VCP/p97 has been shown to be involved in degradation of proteins associated with the outer mitochondrial membrane and chromatin, soluble proteins and protein aggregates (Figure 5) (Meyer, 2012, Stolz et al., 2011). Although mitochondria have mechanisms to eliminate misfolded and unwanted proteins, proteins at the cytosolic side of the outer mitochondrial membrane undergo ubiquitination and proteasomal degradation. Furthermore, VCP/p97 has been shown to be involved in proteasome-independent degradation in the lysosome by regulating endosomal sorting and autophagy (Figure 5) (Meyer, 2012, Stolz et al., 2011).

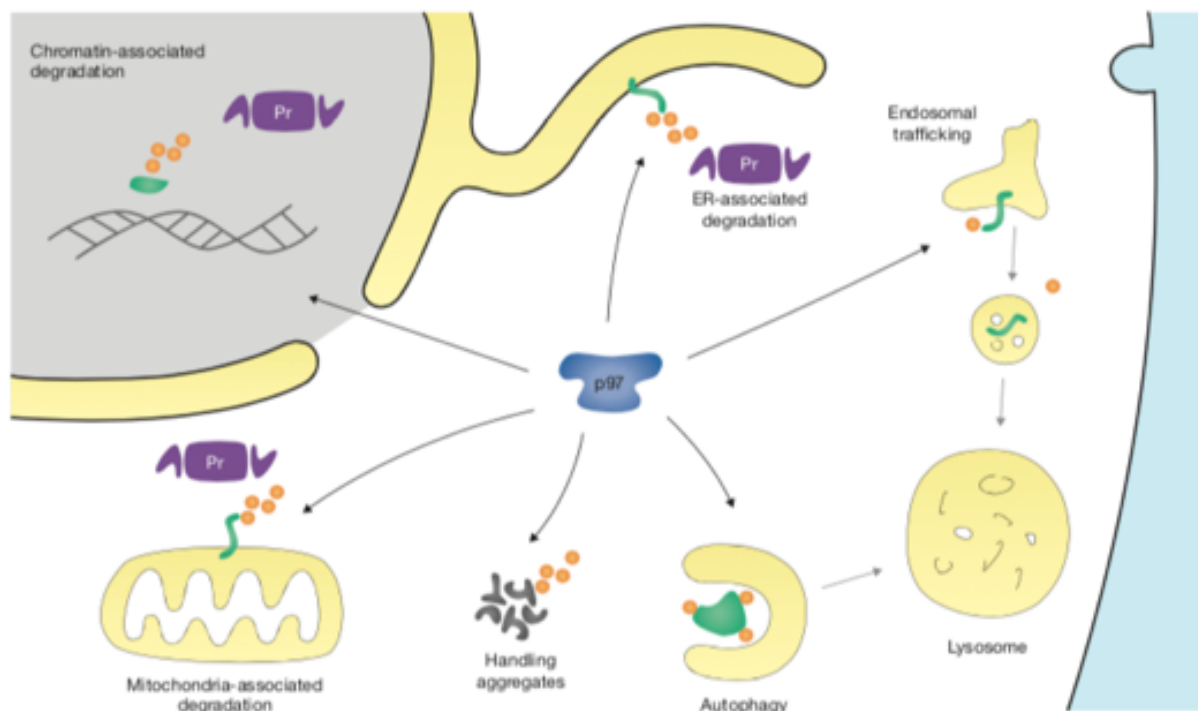


Figure 5. Emerging cellular roles of VCP/p97. VCP/p97 regulates proteasome-mediated degradation of outer mitochondrial membrane proteins, as well as proteins associated with chromatin. VCP/p97 is also involved in the degradation of cytosolic proteins and handling of protein aggregates. These activities help eliminate misfolded proteins to protect the cell from protein stress. Moreover, they help remove regulatory proteins in various intracellular signalling pathways, including those that regulate chromatin remodelling and DNA replication and repair. VCP/p97 facilitates proteasome-independent degradation in the lysosome by regulating protein sorting in the endocytic pathway and autophagy.

Key: Green - target substrates; Pr - proteasome; orange - ubiquitin.

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1.4.4.4 ***Diverse function of VCP/p97***

In addition to the well-established role of VCP/p97 in ubiquitin-dependent protein degradation by the proteasome, further studies have revealed other functions of VCP/p97 that do not involve proteostasis, that include cell cycle regulation, post-mitotic spindle disassembly, homotypic membrane fusion and chromatin-associated functions. It is thought that VCP/p97 is involved in the degradation of cell cycle regulators and evidence suggests that VCP/p97 may regulate the stability of Cyclin E, the G1-cyclin-dependent kinase inhibitor Far1, and proteins that control chromosome segregation during mitosis (Ye et al., 2017). Moreover, Cao and colleagues have reported a connection between VCP/p97 and mitosis. They confirmed that the VCP/p97- Ufd1-Npl14 complex is required for spindle disassembly during mitotic exit (Cao et al., 2003). It is thought that VCP/p97 is required at multiple stages during cell cycle progression but the exact relationship between substrate, VCP/p97 and various cell cycle regulators is yet to be established.

VCP/p97 is closely related to NSF (*N*-ethylmaleimide sensitive factor), a AAA ATPase involved in the fusion of vesicles with their target membranes in the secretory pathway (Meyer et al., 2005). VCP/p97 along with its associated proteins, Ufd1-Npl4, and p47 is involved in the reassembly of the nuclear envelope during mitosis, which requires fusion of vesicles docked on the chromatin (Hetzer et al., 2001). VCP/p97 also mediates the reformation of the Golgi complex following mitotic disassembly (Ye et al., 2017). Furthermore, the p97-Ufd1-Npl4 core complex has been shown to be essential in chromatin-associated processes that ensure correct transcription, replication and stability of the genome (Meyer, 2012).

1.4.4.5 ***VCP/p97 and disease***

VCP/p97 has a wide range of functions suggesting a diverse involvement in disease. VCP/p97 mutations have been associated with a late-onset multisystem degenerative disease that comprises Paget's disease of bone, inclusion body myopathy, and fronto-temporal dementia (IBMPFD), and with several other diseases of the nervous and muscular system including 1-2% of amyotrophic lateral sclerosis (ALS) cases (Johnson et al., 2010).

IBMPFD is an autosomal dominant disorder associated with thirteen mutations in the D1 and N-domains of VCP/p97. These mutations result in elevated ATPase activity and significant conformational alterations of the N-terminal domain (Stolz et al., 2011). These structural alterations cause an imbalance in cofactor binding and accumulation of ubiquitylated proteins associated with VCP/p97. However, it is unknown how these changes relate to the pathogenesis of IBMPFD even though it is believed to be linked to problems with protein quality control (Chapman et al., 2011). Studies using disease models in cell lines (Gitcho et al., 2009) and mice (Custer et al., 2010) have shown, that loss of VCP/p97 function through mutation or depletion results in ER stress, ER expansion, mitochondrial malfunction, production of reactive oxygen species, caspase activity and apoptosis. The first evidence for VCP/p97's connection with autophagy came from studies on the pathogenesis of IBMPFD when VCP/p97 mutations that lead to the formation of intracellular inclusions with protein aggregates were identified (Ju and Weihl, 2010, Meyer, 2012, Tresse et al., 2010). Tresse and colleagues demonstrated that VCP/p97 is essential for ubiquitin containing autophagosome maturation and defects in this function may be a contributory factor to the pathogenesis of IBMPFD (Tresse et al., 2010). However,

the specific process of quality control altered in IBMPFD is yet to be established. Some studies suggest that both the UPS and autophagy are disturbed (Ju and Weihl, 2010), other studies indicate only autophagy to be perturbed (Tresse et al., 2010). Despite of the exact mechanism, VCP/p97 is essential for cellular proteostasis and maintains skeletal and neurological health.

Increased VCP/p97 expression has also been linked with poor prognosis of certain cancers including prostate cancer, non-small cell lung carcinoma, pancreatic cancer, breast cancer and leukaemia (Braun and Zischka, 2008, Chapman et al., 2011, Haines, 2010). VCP/p97 has been implicated in the direct regulation of some key cancer proteins including hypoxia inducible factor-1 α (HIF1 α), a tumour angiogenesis and metastases promoter, Aurora B kinase which is overexpressed in cancer cells and implicated in genomic instability, and neurofibromatosis type 1 (NF1), a tumour suppressor and inhibitor of Ras signalling (Haines, 2010). Another strong link between VCP/p97 and cancer exists through nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B) signalling. NF κ B is a protein complex that controls transcription of DNA, cytokine production and cell survival playing a key role in regulating the immune system and infection. VCP/p97 has been demonstrated to interfere with this signalling pathway, resulting in upregulation of NF κ B resulting in increased cell survival and proliferation (Chapman et al., 2011).

Although the pathogenesis of VCP/p97-related diseases remains to be established, their associated cellular dysfunction has been linked to defective proteostatic fine-tuning (Ju and Weihl, 2010, Meyer and Weihl, 2014). This putative mechanism is compatible with the notion that relatively minor but chronic or intermittent imbalances in proteostasis contribute to many age-related diseases (Meyer and Weihl, 2014, Schneider and Bertolotti, 2015b).

However, perturbations in fine-tuning that result in chronic or intermittent imbalances in intracellular proteostasis are extremely challenging to replicate *in vitro* and in particular *in vivo* models.

1.4.4.6 **VCP/p97 inhibitors**

The importance of VCP/p97 in health and disease is evident. VCP/p97's role in mediating protein degradation of ubiquitinated proteins is well studied. More recent studies have revealed its role in the degradation of aggregated proteins through the aggresome-autophagy pathway. Given the success of the proteasome inhibitor bortezomib for the treatment of multiple myeloma, it seems that targeting other components of the UPS such as VCP/p97 could offer alternative cancer treatment pathways. However, the value of VCP/p97 as a target in cancer treatment is subject to the development of additional pharmacological tools to modulate its activity as well as better understanding of its multiple cellular functions (Meyer, 2012).

The first available VCP/p97 inhibitor was Eeyarestatin 1 reported to block ERAD by binding to the D1 domain of VCP/p97 (Fiebiger et al., 2004). Further efforts lead to the discovery of a small molecule $N2,N^4$, dibenzylquinazoline-2-4-diamine (DBeQ) by Chou and colleagues (Chou et al., 2011b, Chou and Deshaies, 2011). DBeQ is a selective, potent, reversible, ATP-competitive inhibitor of VCP/p97 that showed IC_{50} value $<10\mu M$ in the ATPase activity assay that has proven useful as a research tool and as a potential starting point for the development of novel cancer agents (Auner et al., 2013, Chou et al., 2011b, Chou and Deshaies, 2011). DBeQ exerts its effects by blocking protein degradation processes known to depend on VCP/p97; ubiquitin fusion degradation, endoplasmic-reticulum associated-degradation of VCP/p97-

dependant UPS substrates and autophagic degradation by inhibiting both D1 and D2 VCP/p97 ATPase domains, with the D2 domain being the main target (Chou et al., 2011b, Chou and Deshaies, 2011, Ding et al., 2019). DBeQ upregulated CHOP and resulted in cell death suggesting that DBeQ-dependent inhibition of VCP/p97 resulted in lethal ER stress response. DBeQ also induced autophagy, as evidence by the accumulation of LC3 (Chou et al., 2011b, Chou and Deshaies, 2011). Thus, DBeQ interferes with both the ERAD and autophagic protein degradation pathway rapidly inducing cell death. Moreover, experiments using cell lines derived from solid tumours, indicated that DBeQ induces caspase activation and cell death although the exact mechanism is yet to be elucidated (Chou et al., 2011a).

More recently, other VCP/p97 inhibitors such as NMS-873 and CB-5083 have been identified. NMS-873 is a non-competitive inhibitor thought to inhibit VCP/p97 by binding at the D1-D2 interdomain linker and stabilising the D2-ADP-bound form thereby breaking the catalytic cycle (Chou and Deshaies, 2014). CB-5083 is a potent, reversible, and orally bioavailable ATP-competitive VCP/p97 ATPase agent which has been tested in human phase I clinical trials for the treatment of cancer (Anderson et al., 2015, Le Moigne et al., 2017). CB-5083 functions as an inhibitor that binds selectively to the ATP-binding site of the D2 domain. Despite its promising anti-cancer activity, CB-5083 failed its Phase I clinical trial due to an unexpected off-target effect, which requires further improvement of the inhibitor (Tang et al., 2017). More recently, covalent irreversible VCP/p97 inhibitors are being explored. Ding and colleagues (Ding et al., 2019), reported a new chemical inhibitor of VCP/p97, Cys522 thought to offer advantages including superior biochemical efficiency and lower dosage requirement.

As NMS-873, CB-5083 and Cys522 were not available in the first years of my PhD work, and as DBeQ is generally accepted as a highly selective inhibitor of VCP/p97, my experimental work is based on the use of DBeQ rather than any of the other inhibitors.

1.4.5 The proteasome

The proteasome is the main executor of substrate-specific proteolysis in eukaryotic cells. It is a large protein complex responsible for the degradation of proteins via the UPS. The process involves conjugation of multiple ubiquitin molecules to the target protein followed by degradation of the tagged protein into peptides by the proteasome complex (Figure 6 a and b).

1.4.5.1 *Proteasome structure*

The 26S proteasome is an ATP-dependent multi-catalytic complex that degrades ubiquitin-modified proteins into small peptides and consists of a 20S catalytic core and one or two 19S regulatory complexes referred to as caps (Coux et al., 1996, Raule et al., 2014, Voges et al., 1999). The catalytic core is a barrel-shaped structure composed of four heptameric rings; the outer rings are made up of α -subunits (α 1-7) while the two inner rings consist of β -subunits (β 1-7) that contain multiple protease activities that are often labelled as caspase-like (β 1), trypsin-like (β 2) and chymotrypsin-like (β 5) (Figure 6 b). On exposure to inflammatory cytokines such as interferon gamma (IFN- γ) and tumour necrosis factor alpha (TNF α), three constitutive β -subunits can be replaced by homologous core subunits (β 5i/LMP7, β 2i/MECL-1, β 1i/LMP2) that alter the proteolytic specificities of the proteasome in the so-called immunoproteasomes (Figure 6 c) (Goldberg et al., 2002). The 19S regulatory particle is comprised of two eight-subunit subcomplexes, the lid and the base (Figure 6 b). The base contains six proteasomal ATPases attached to the α -ring of the core complex.

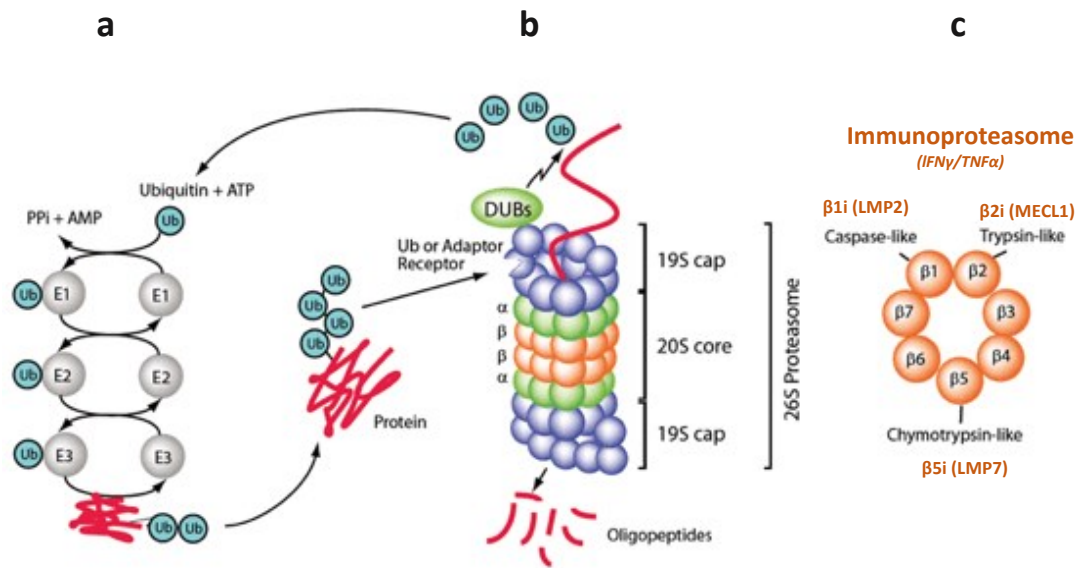


Figure 6. Proteasome structure and function. (a) Protein degradation through the UPS begins with the polyubiquitination (Ub) of target proteins by a three-enzyme cascade (E1, E2 and E3) followed by degradation of the protein into peptides by the 26S proteasome complex. (b) The 26S proteasome is composed of the 20S core catalytic complex capped on both sides by the 19S regulatory complexes or caps. The 20S complex is arranged as a stack of four rings, two α (green) and two β (orange), organised in the structure $\alpha \beta \beta \alpha$. Both α and β rings are composed of seven subunits. Thus, the general structure of the complex is $\alpha_{1-7} \beta_{1-7} \beta_{1-7} \alpha_{1-7}$. The three catalytic sites: the trypsin-, chymotrypsin-, and caspase-like reside on different β rings. The α subunits are catalytically inactive playing a role in the binding of the 19S regulatory complexes. The base of the 19S regulatory particle is associated with ATPases thought to unfold target proteins that are translocated through a gated channel into the 20S catalytic core. (c) Exposure to cytokines such as IFN- γ and TNF α , can result in the replacement of three constitutive β -subunits by homologs core subunits ($\beta 5i$ /LMP7, $\beta 2i$ /MECL-1, $\beta 1i$ /LMP2) that alter the proteolytic specificities of the proteasome in the so-called immunoproteasome

These ATPases (Rpt1-6) are present in the base together with three non-ATPases subunits (Rpn1, -2 and -10). Direct interactions have been reported between these ATPases and the α -subunits of the core complex. The ATPases in the base probably unfold substrates and translocate them through a gated channel into the 20S catalytic core (Figure 6 b) (Braun et al., 1999, Groll et al., 1997, Horwich et al., 1999). The lid of the regulatory particle is made up of the eight remaining ATPase subunits (Rpn3, -4, -5, -6, -7, -8, -9, -11, -12) that can be released from the proteasome or rebind under certain conditions. The role of the lid is still unclear, although it is thought to be necessary for proper degradation of polyubiquitinated proteins (Glickman et al., 1998).

1.4.5.2 ***Proteasome function***

Proteins earmarked for degradation by polyubiquitination are delivered to the proteasome by VCP/p97 and recognized by the 19S particle that also facilitates protein deubiquitination, unfolding and translocation into the 20S proteasome core particle (Kwon and Ciechanover, 2017, Meyer, 2012, Tsai and Weissman, 2011). The mechanism of proteolysis by the β subunits of the 20S core particle is a threonine-dependent process. Although the three catalytic β subunits have a common mechanism, they have slightly different specificities but all three possess a conserved lysine residue required for proteolysis. Degradation of the target protein occurs within the central pore formed by the association of two β rings. The proteasomal products of the proteolytic processes are small peptides normally 7-9 residues long, that are further broken down by aminopeptidases into amino acids (Glickman and Ciechanover, 2002).

While the proteasome degrades proteins of diverse cellular origin, its best understood role is in ERAD (Wu and Rapoport, 2018). Immunoproteasomes play additional roles that are essential for adequate immune responses in that they generate MHC class I ligands (Kincaid et al., 2012) and have specialised functions including high-rate hydrolysis of histones, suggesting particular roles in the regulation of gene expression (Raule et al., 2014).

1.4.5.3 ***Proteasome inhibitors***

Proteasome inhibitors (PIs) were initially used as tools to study the cellular roles of the proteasome. Observations that they preferentially kill leukaemia and lymphoma cells *in vitro* and *in vivo* rapidly triggered their development into clinical agents (Kumatori et al., 1990, Orlowski et al., 1998). The boronate bortezomib, which was first approved by the Food and Drug Administration for treatment of multiple myeloma (MM) in 2003, is a reversible inhibitor predominantly of the chymotrypsin-like activity of both the constitutive ($\beta 5$) and the immuno-proteasome (LMP7) (Figure 6 c) (Crawford and Irvine, 2013). Other proteasome inhibitors currently approved for clinical use are ixazomib, and carfilzomib that irreversibly binds to $\beta 5$ and LMP7 (Gandolfi et al., 2017). All three approved PIs are heavily used in a variety of drug combinations in clinical practice and ongoing clinical trials.

Several potential mechanisms of action have been proposed for the toxic effects of PIs on MM cells. They include, but are not limited to, induction of p53 and activation of c-JUN N-terminal kinases (JNK) (Hideshima et al., 2003), diminished pro-survival signalling by NF- κ B (Ma et al., 2003), impairment of genotoxic stress response mechanisms (Mitsiades et al., 2003), oxidative stress (Campanella et al., 2013, Fink

et al., 2016), and dysregulation of members of the B-cell lymphoma 2 (Bcl-2) family of proteins (Fennell et al., 2008, Hu et al., 2012). However, probably the most abundant evidence points to a key role of PI-induced ER stress (Lee et al., 2003, Meister et al., 2007, Wang et al., 2009). The notion that PIs kill MM cells by disrupting the degradation of toxic misfolded proteins, presumed to derive from high level immunoglobulin production, provides an appealing explanation for why MM cells are so exquisitely sensitive to PIs. It is also compatible with observations on differences between PI-sensitive and -resistant MM cells, in particular the so-called 'load versus capacity' model that predicts that cells with a high degradative workload (misfolded proteins) and low proteasomal capacity (cellular proteasome content) will be sensitive to PIs, and *vice versa* (Bianchi et al., 2009, Cenci et al., 2012). The model is also conceptually supported by several studies that link UPR capacity or proteasomal recovery mechanism with levels of PI sensitivity (Chaidos et al., 2013, Leung-Hagesteijn et al., 2013, Ling et al., 2012, Michallet et al., 2011, Radhakrishnan et al., 2010, Sha and Goldberg, 2014, Steffen et al., 2010).

1.5 Bone homeostasis

Bone is a dynamic tissue with several important functions including mechanical support of soft tissues, levers for muscle action, protection of the central nervous system, storage of minerals, and housing and support of hemopoiesis (Rodan, 1998). There are two types of bone; cortical which provides mechanical function and is protective, and trabecular which provides strength and metabolic function (Feng and McDonald, 2011).

Bone homeostasis referred to as bone remodeling, is maintained by an active process of bone formation by osteoblasts and bone resorption by osteoclasts (Nakashima, 2016). The balance between bone formation and resorption is vital for sustaining bone mass and mineral homeostasis. Bone remodeling requires vitamins D, C, and A plus minerals such as calcium, phosphorous, and magnesium. Hormones such as parathyroid hormone, growth hormone, and calcitonin are also essential for bone growth and maintenance (Siddiqui and Partridge, 2016). Moreover, the ability of bone to modify its structure and adapt to mechanical load implies that mechanical load can regulate bone remodeling; heavy loads increasing bone formation and decreasing resorption leading to thicker and stronger bones and vice versa (Rodan, 1998).

1.5.1 Osteoblast differentiation and function

Mesenchymal stromal cells (MSCs) are multipotent adult stem cells first discovered by the Friedenstein's group in bone marrow stroma as fibroblast-like cells with the capacity to differentiate into various cell types including osteoblasts, chondrocytes, myocytes, and adipocytes (Horwitz, 2006). Previous studies have shown that MSCs play an important role during bone formation and bone remodeling and have been identified as potential sources for many clinical applications (Abdallah and Kassem, 2009, Salem and Thiernemann, 2010, Zou et al., 2009).

Bone develops through a tightly regulated process that results in a hierarchically ordered, three-dimensional structure. New bone is synthesized by specialized terminally differentiated osteoblasts (OBs). OBs are derived from MSCs in a step-wise differentiation process that consists of proliferation, matrix maturation, and matrix mineralisation. This differentiation process is regulated by transcription factors, cytokines, and co-regulatory proteins.

The key molecular switch in the commitment of mesenchymal progenitors to OB lineage is runt-related transcription factor 2 (RUNX2, also known as core-binding factor alpha-1, Cbfa-1), a master transcription factor of OB differentiation that has multiple upstream regulators including the Wnt/Notch system and a wide variety of targets. Following RUNX2, Osterix (Osx, also known as transcription factor Sp7), a zinc-finger-containing transcription factor, influences cell fate decision in OB lineage (Ducy et al., 1997, Horiuchi et al., 2016, Nakashima, 2016). Thus, RUNX2 and Osterix are responsible for initiating and regulating early osteogenesis and late mineralisation (Garrett et al., 2003). Furthermore, ATF4, a downstream target of PERK, plays a crucial role in maintaining OB

function (Garrett et al., 2003, Horiuchi et al., 2016, Rutkovskiy et al., 2016).

Mature osteoblasts synthesize large quantities of ECM proteins, the most abundant is type I collagen. However, OBs also produce non-collagenous proteins involved in mineralisation, such as osteocalcin (also known as bone gamma-carboxyglutamate protein, *BGLAP*) and osteonectin. Mature osteoblasts ultimately become part of the mineralized matrix and they turn into osteocytes or bone-lining cells (Terpos et al., 2018). To handle the prominent secretory function, OBs have a well-developed ER and Golgi apparatus (Figure 7), similar to that observed in plasma cells and pancreatic β cells (Horiuchi et al., 2016).

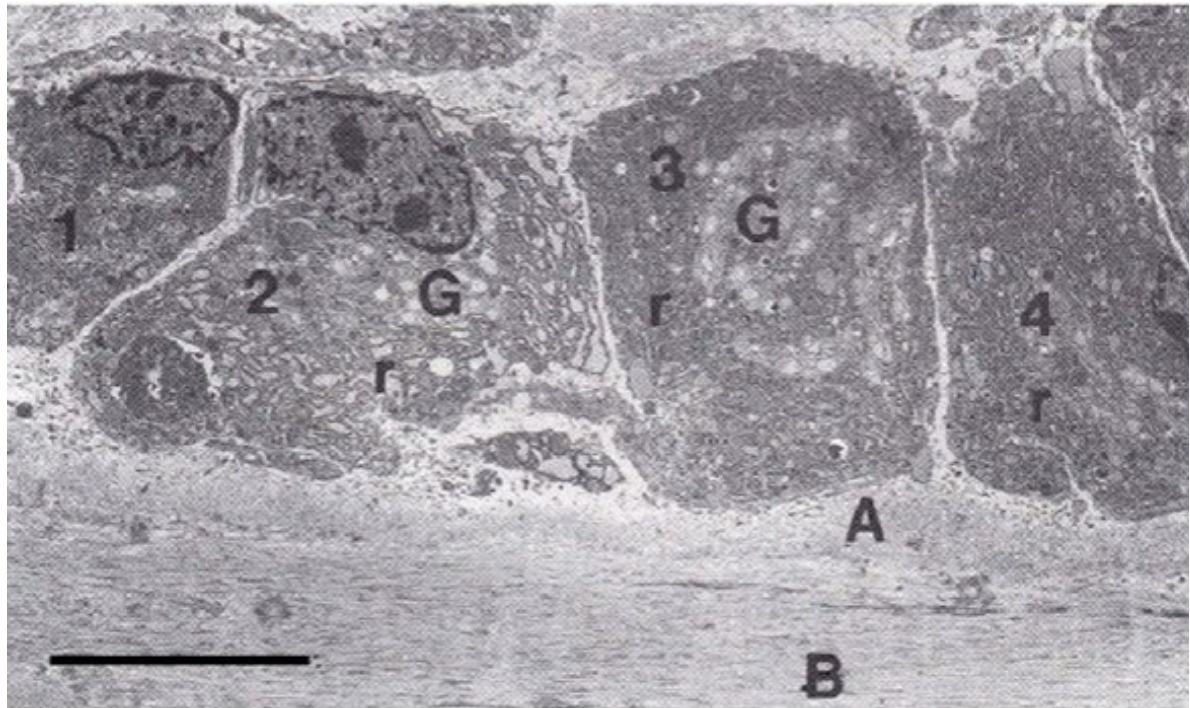


Figure 7. Osteoblasts are secretory cells. Transmission electron micrograph of osteoblasts (numbered) on bone surface in which the collagenous matrix has been deposited in two layers (A and B). The Golgi apparatus (G) and rough endoplasmic reticulum (r) are prominent organelles in osteoblasts. Original magnification x2800, bar 0.1 μm .

Reproduced, with permission, from Andrades et al., Bone Engineering: A Matter of Cells, Growth Factors and Biomaterials (2013). Open access peer-reviewed chapter, DOI: 10.5772/56389.

1.5.2 The UPR and osteoblast differentiation

OBs secrete large quantities of proteins to generate and maintain the skeletal system. To cope with this secretory function, OBs must acquire capacity for protein synthesis and machinery for the quality control and transport of newly secreted proteins. Several studies have demonstrated the UPR to play a crucial role during differentiation of OBs to achieve this goal. Moreover, studies in the past few years, have revealed that the UPR has wider functions in skeletal development than initially thought, coordinating many stages of skeletal development and bone homeostasis (Horiuchi et al., 2016).

PERK pathway activation has been demonstrated to be required for OB differentiation, proliferation and function (Wei et al., 2008). ATF4, which is induced downstream of PERK, is an essential transcription factor for OB differentiation and bone formation through the regulation of collagen synthesis (Saito et al., 2011). PERK^{-/-} mice are severely osteopenic, which is caused by a reduced number of mature OBs, impaired OB differentiation and reduced type 1 collagen secretion (Saito et al., 2011, Wei et al., 2008). Impaired differentiation of OBs in PERK KO mice, was associated with decreased expression of RUNX2 and Osterix. PERK^{-/-} osteoblasts exhibited decreased alkaline phosphatase activities and delayed mineralised nodule formation relative to wild type cultures (Saito et al., 2011).

A study by Tohmonda and colleagues demonstrated the IRE pathway and its transcription factor XBP1 to be required for bone morphogenetic protein 2 (BMP2) induced OB differentiation. Furthermore, they identified Osterix as a target gene of XBP1. This study demonstrated the UPR to have two functions during OB differentiation; expansion of the capacity of the ER to handle the increased

production of ECM proteins and stimulation of Osterix expression through the IRE-XBP1 pathway to promote maturation of OBs (Tohmonda et al., 2011).

AFT6 has been shown to mediate BMP2 induced OB differentiation and upregulation of osteogenic genes. Results from experiments by Jang and colleagues suggested that BMP2 induces OB differentiation through RUNX2-dependent ATF6 expression which directly regulates osteocalcin transcription (Jang et al., 2012).

In summary, the high secretory function of OBs, challenges proteostasis and explains why both the UPR and mechanisms of protein degradation play a crucial role during the differentiation and for the function of these cells (Murakami et al., 2009, Tohmonda et al., 2011).

1.6 Bone composition and physical properties

The composition of bone is approximately 10% cells, 60% mineral crystals, and 30% organic matrix (Feng and McDonald, 2011). The mineralised matrix of bone has an organic component of mainly collagen with other biomolecules including proteoglycans and non-collagenous proteins, and an inorganic component of mineral salts primarily calcium and phosphate. Initially, mineral is amorphous, but with maturation calcium and phosphate bind to form hydroxyapatite ($\text{Ca}_5\text{PO}_4)_3(\text{OH})$) in the form of rod-shaped crystals (Von Euw et al., 2019). Carbonate is also present as calcium carbonate and carbonate apatite.

Mineralisation is responsible for the rigidity and compressive strength of bone. Conversely, collagen fibrils have high elasticity, little compressive strength and considerable tensile strength. Although seemingly stiff, bone is elastic which is important for function and being able to withstand impact. Differences in the mineral-collagen ratio leads to physical properties changes; less mineral resulting in greater flexibility and more mineral in increased brittleness. This is important as bone's susceptibility to fracture is dependent on the microarchitecture and physiochemical composition (Akkus et al., 2004). Thus, bone tissue quality is dependent on the relative quantities and physiochemical properties of its main components, the organic matrix and the mineral crystals.

1.7 Aim of the project

Intracellular protein homeostasis (proteostasis) is essential for the maintenance of normal cellular function and the preservation of tissue integrity and health. Impaired proteostasis, is considered to play an important role in the pathogenesis of a wide range of human diseases, many of which are linked to ageing. These include but are not limited to multisystem degenerative disorders, diabetes, and cancer. How proteostasis is maintained in healthy cells and lost in diseased cells is central to understanding the mechanisms of these common diseases. Moreover, drugs that perturb proteostasis by interfering with cellular mechanisms that control either protein synthesis or degradation are being used increasingly for the treatment of diseases that are linked to aberrant proteostatic regulation. However, how impaired intracellular proteostasis affects complex tissues rather than individual cells is poorly understood.

The overall aim of this project is to use an *in vitro* model of bone-like tissue formation to study how mild perturbations of intracellular proteostasis (caused by pharmacological interference with protein degradation) affect biochemical and biophysical tissue properties. I also aim to combine an array of tissue characterisation techniques to determine if it may be feasible to develop *in vitro* platforms that allow for the functional effects of proteostasis imbalances on tissue to be evaluated quantitatively in a model that could be used for high-throughput pre-clinical drug screening purposes.

2 Materials and Methods

2.1 Human mesenchymal stromal cell (hMSC) culture

Human samples used in this research project were obtained from the Imperial College Healthcare Tissue Bank (ICHTB, HTA license 12275). ICHTB is supported by the National Institute for Health Research (NIHR) based at Imperial College Healthcare NHS Trust and Imperial College London. ICHTB is approved by the UK National Research Ethics Service to release human material for research (12/WA/0196), and samples for this project were issued from sub-collection R16052.

Bone marrow aspirates from healthy paediatric donors were obtained at St. Mary's Hospital Imperial College Healthcare NHS Trust (IHCNT) for the purpose of allogeneic haematopoietic stem cell transplantation for a variety of diseases. hMSC were routinely cultured and expanded for clinical use for the treatment of Graft-versus-Host-Disease (GvHD) in patients post allogeneic haematopoietic stem cell transplantation. Written informed consent for the use of surplus hMSC for research was obtained from the donor's parents in accordance with institutional guidelines.

Primary cultures were established using CellSTACK® culture chambers (Corning Incorporated, Life Sciences), filling caps (Corning Incorporated, Life Sciences) and Macopharma seeding sets (Macopharma) in a Grade A cleanroom environment under good manufacturing practice (GMP) conditions (Figure 8).

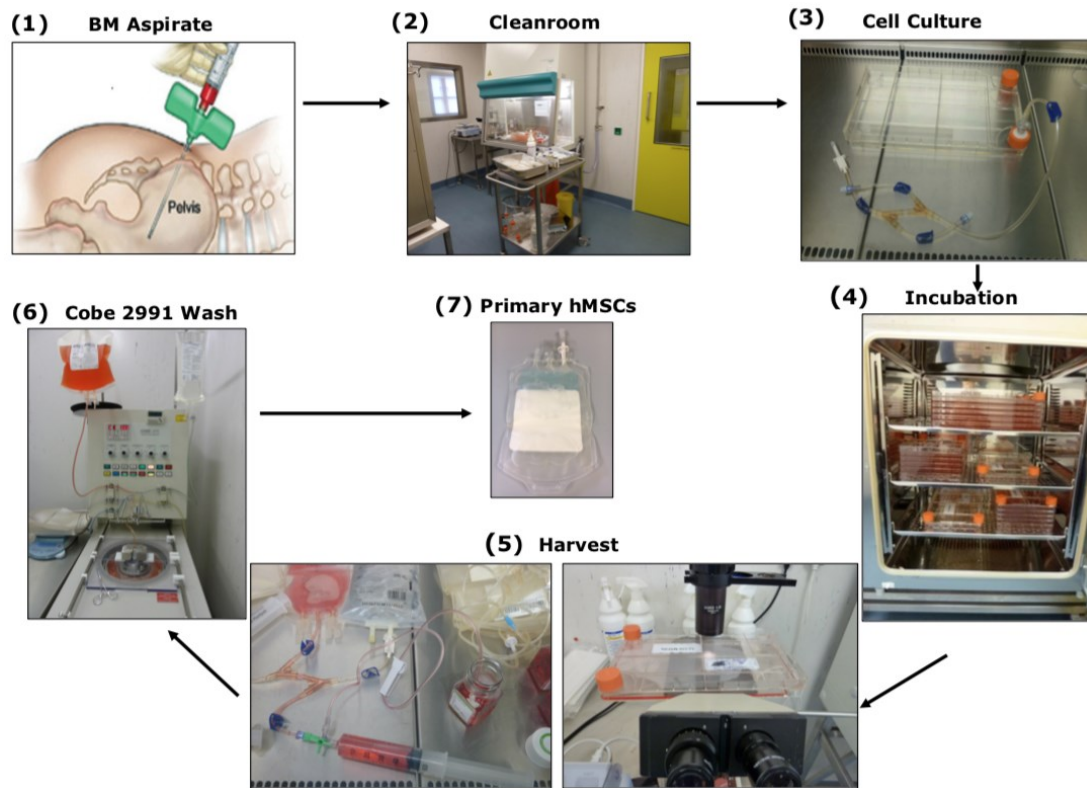


Figure 8. Schematic depicting the expansion of hMSC for clinical use. (1) Bone marrow is taken from paediatric donors. (2) The bone marrow is transported to the laboratory for processing in a sterile Grade A cleanroom environment. (3) In the cleanroom, bone marrow cells are seeded in sterile culture chambers and (4) cultured at 5% CO₂ at 37°C until confluent. At confluency, cells are (5) harvested by trypsinising for detachment, (6) washed using the Cobe 2991 device and (7) aliquoted into bags for clinical use.

I targeted a seed rate of 25×10^6 total nucleated cells per level of the culture chamber. Cells were cultured in Minimum Essential Medium α with GlutaMAX™ (α -MEM, Gibco) supplemented with 5% pooled human platelet lysate, the standard concentration used for MSC optimal growth (Becherucci et al., 2018, Burnouf et al., 2016, Doucet et al., 2005). Platelets were obtained by apheresis from 10 donors by the National Blood Service were pooled, centrifuged at 3000 rpm for 10 minutes to eliminate platelet bodies, and frozen at -80°C . Platelet lysate pools were batch-tested for their ability to support hMSC growth.

Cells were cultured in a humidified incubator in 5% CO_2 at 37°C until confluent. When confluent, usually after 14 days, cells were harvested and cryopreserved using a controlled rate freezer in 5% human albumin solution (Biotest) containing 10% dimethylsulfoxide DMSO (CryoPur™, OriGen) in a 1:1 ratio. Prior to cryopreservation, hMSCs were immunophenotyped by staining with the standard reference panel of labelled mouse anti-human antibodies (CD105-APC, CD73-PE, CD90-APC, CD45-FITC, CD34-PE, CD3-APC, CD19-PE, HLA-DR-FITC and CD14-PE; BD Biosciences) as defined by the International Society of Cellular Therapy (ISCT) (Dominici et al., 2006) using a FACS Canto II analyser (BD Biosciences) using a FACS Diva software. hMSC expressed CD90, CD105 and CD73 and were negative for hematopoietic markers and CD45), T-cell markers (CD3) and myeloid markers (CD14 and CD19) (CD34 (Figure 9) (Dominici et al., 2006, Lv et al., 2014, Becherucci et al., 2018).

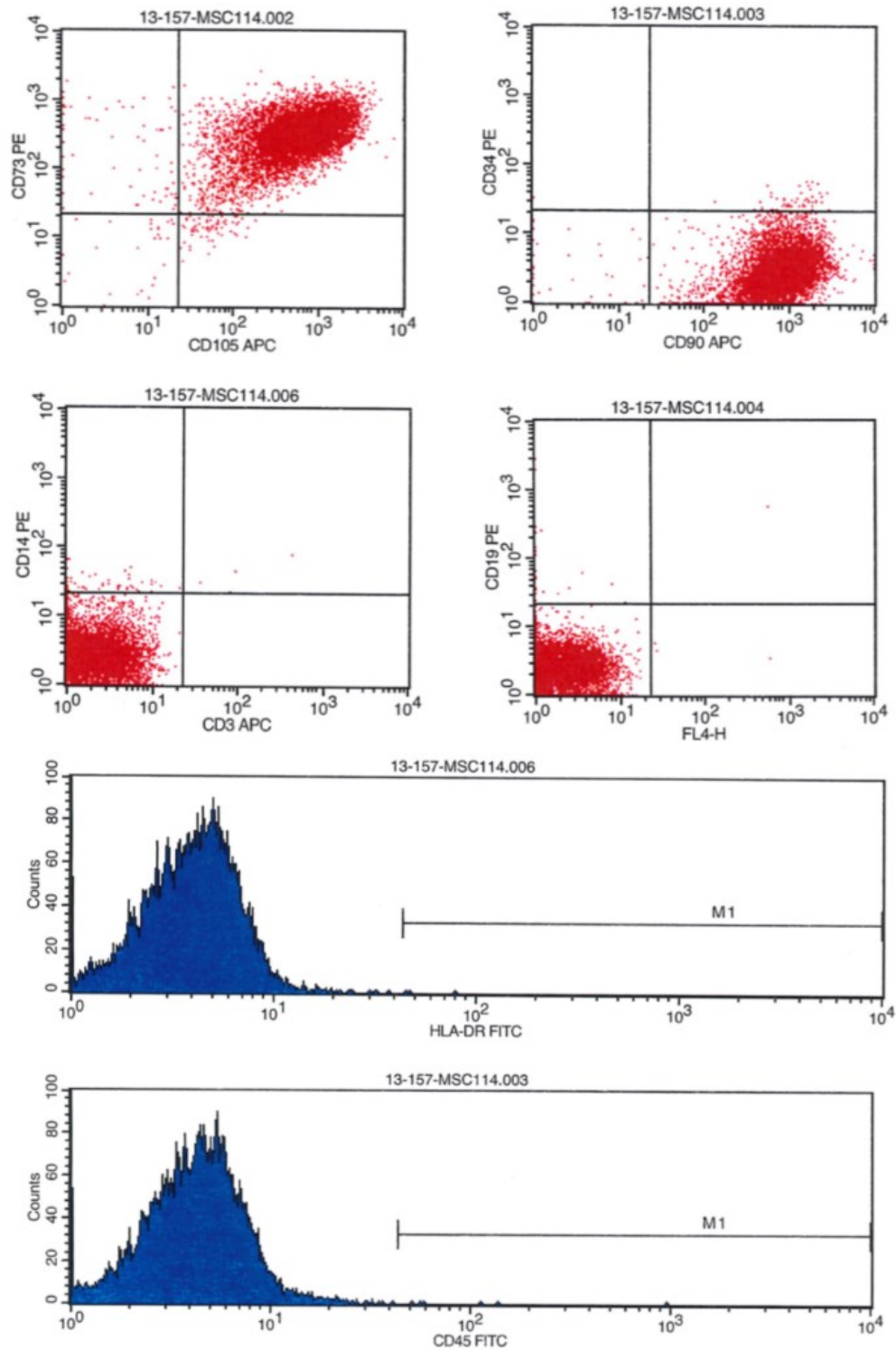


Figure 9. Immunophenotype of primary human MSCs. Representative flow cytometry analysis demonstrating the CD73⁺ CD105⁺ CD90⁺ CD34⁻ CD14⁻ CD3⁻ CD19⁻ HLA-DR⁻ CD45⁻ phenotype of established MSC cultures.

For experiments on undifferentiated hMSCs described in this thesis, cells were grown in α -MEM supplemented with 10% heat-inactivated foetal bovine serum (FBS, reserve number 3238056, Sigma), 1000 U/mL penicillin, and 10 mg/ml streptomycin (Sigma). Medium was changed every 3 to 4 days, and cells were used until passage 8 only.

2.2 Osteogenic differentiation of hMSCs and inhibitors

hMSCs were seeded at 5×10^3 cells/cm² in α -MEM. After 24h, cells were approximately 80% confluent, and osteogenic differentiation was initiated by switching to commercially available Stempro® Osteogenesis Differentiation kit medium (Invitrogen) or in house prepared standard osteogenic differentiation medium ((Birmingham et al., 2012, Hanna et al., 2018) consisting of α -MEM, 15% FBS, 100 μ M ascorbic acid (Scientific Lab Supplies), 2.5 mM β -glycerophosphate (Calbiochem) and 100 nM dexamethasone (Sigma). Medium was changed every 3 to 4 days.

VCP/p97 and the proteasome were inhibited with DBeQ (BioVision) or bortezomib (Calbiochem). Tunicamycin was purchased from Sigma.

2.3 Cell lines

Human osteosarcoma Saos-2 cells (obtained from Eileen Gentleman from King's College London, UK) and human OPM-2 multiple myeloma cells (obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen DSMZ) underwent identity confirmation using short tandem repeat profiling of 10 loci (Core Genomic Facility, Medical School, University of Sheffield) and

were cultured in RPMI-1640 (Invitrogen, UK) supplemented with 10% FBS. Cells were intermittently tested for mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Lonza).

2.4 RNA extraction and reverse transcription

Cells were harvested and then snap frozen in liquid nitrogen. RNA was extracted using the GeneJET RNA Purification kit (Thermo Scientific) followed by removal of genomic DNA according to the manufacturer's instructions. Remaining traces of DNA were removed using DNase I treatment (Invitrogen). cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific) according to the manufacturer's instructions using an Applied Biosystems 2720 Thermal Cycler (Life Technologies).

2.5 Quantitation of gene expression by real-time polymerase chain reaction (PCR)

PCR reactions were performed on an Applied Biosystems StepOnePlus™ PCR machine using 10 µl SYBR Green JumpStart™ Taq ready Mix™ PCR (Sigma), 0.48 µL sequence-specific primers at 300 nM (Supplementary Table 1) and 5 µL cDNA.

A three-step cycle was employed: (1) denaturation at 94°C for 2 minutes; (2) annealing/extension at 60°C for 1 minute; (3) melting from 60°C to 94°C for 2.5 minutes. The $\Delta\Delta C_T$ method was used to quantify fold changes in expression ($2^{-\Delta\Delta C_q}$) of each gene of interest and normalised to the expression of *GAPDH* in undifferentiated control cells.

2.6 Alizarin Red S staining

Mineralisation was measured by staining with Alizarin Red S (ARS), a Ca^{2+} -binding dye. Cells were washed with phosphate-buffered saline (PBS; Sigma) and fixed with 2 ml of 10% formalin in PBS for 20 minutes. Cells were washed twice in PBS followed by staining with 2% (w/v) Alizarin Red S (Sigma) solution in dH_2O (after adjusting the pH to 4.2 using 10% NH_4OH) for 10 minutes at room temperature. The dye solution was drained, and the cells washed with running water for 30 minutes. Cultures were visualised using an EVOS x1 Core digital cell imaging microscope system.

2.7 Alkaline Phosphatase assay

Alkaline phosphatase (ALP) activity was measured using the p-Nitrophenyl Phosphate Liquid Substrate system (Sigma). Briefly, approximately 2×10^5 cells were rinsed with PBS and then lysed with 500 μl of lysis solution made up of 0.09% Triton X-100 in dH_2O . After 30 minutes, 50 μl of the lysed sample was mixed with 50 μl of ALP substrate in one well of a 96 well plate. The remainder of the lysed sample was kept for measurement of protein content. The reaction was stopped after 30 minutes by adding 50 μl of 3M NaOH per well. The absorbance was then read on the colorimetric FLUOstar Omega BMG Labtech plate reader at 405 nm. ALP activity was normalised to protein content of the sample, which was measured using the colorimetric Pierce™ BCA Protein Assay kit (Thermo Scientific). Briefly, 25 μl of lysed sample were transferred into a well of a 96-well-plate. 200 μl of BCA reagent was added to each well, followed by incubation of the plate for 30 minutes at 37°C. Absorbance was

then measured at 562 nm using a FLUOstar Omega BMG Labtech plate reader.

2.8 Calcium quantification

Differentiated cell cultures were rinsed with PBS and then incubated at 4°C on an orbital shaker overnight with 1 ml of 0.5M HCl to dissolve calcium from the bone nodules. Calcium quantification was then performed on lysates using the Calcium Colorimetric Assay kit (BioVision) according to the manufacturer's instructions. Briefly, 10 µl of sample (HCl with dissolved nodules) or known standards were incubated with 90 µl of chromogenic reagent that binds to the complex formed between calcium ions and 0-cresolphthalein, and 60 µl of assay buffer. The absorbance was then measured at 575 nm using a FLUOstar Omega BMG Labtech plate reader. The calcium concentration in the samples was then calculated using the standard curve of serial dilutions (0 to 2 µg).

2.9 *In vitro* mineralisation assay

Mineralisation was measured quantitatively using the OsteoImage™ mineralisation assay (Lonza) according to the manufacturer's instructions. The assay is based on specific binding of the fluorescent OsteoImage™ staining reagent to the hydroxyapatite portion of bone-like nodules deposited by cells. Briefly, the plate was removed from the incubator and allowed to cool down to room temperature and then rinsed with PBS. Cells were fixed by incubating for 20 minutes with 200 µl of ethanol followed by rinsing twice with 200 µl of wash buffer. 100 µl of diluted staining reagent

was added per well and the plate was incubated at room temperature for 30 minutes, protected from light followed by three washes with 200 μ l diluted wash buffer. Fluorescence (excitation/emission wavelengths 492/520) of the sample was measured using a FLUOstar Omega BMG Labtech plate reader.

2.10 Cellular metabolic activity (viability)

Cellular metabolic activity as a read-out of cellular viability was measured using the Alamar Blue® (Life Technologies) assay according to the manufacturer's instructions. The assay is based on the reduction of resazurin, a weakly fluorescent blue dye, to highly fluorescent red resorufin in healthy cells. The Alamar Blue® cell viability reagent was incubated at 10% of sample volume for 1-4 hours at 37°C, and the fluorescence (590 nm) of the sample measured using a FLUOstar Omega BMG Labtech plate reader. Viability was determined relative to untreated cells or cells treated with vehicle only.

2.11 Immunoblotting

Whole-cell protein extracts were prepared on ice using lysis buffer (Cell Signalling) supplemented with Complete EDTA-free Protease Inhibitor Cocktail (Roche). Then, after 10 minutes on ice and centrifugation at 4°C (14,000 rpm) for 10 minutes, the supernatant was collected. Protein concentration was measured using Bradford reagent (Bio-RAD) according to the manufacturer's instructions. Proteins were denatured at 100°C for 5 minutes with standard SDS-PAGE Loading Buffer (200 mM Tris-Cl, pH 6.8), 400 nM DTT, 8% SDS,

0.4% bromophenol blue, 40% glycerol). Proteins were separated on a 10% SDS polyacrylamide gel using electrophoresis and transferred to PVDF membranes (GE Healthcare). Membranes were blocked with 0.1% TBST (Tris-Buffered Saline, Tween20) containing 5% non-fat milk for 1 hour at room temperature, incubated with primary antibodies against ubiquitin (Cell Signalling Technology, catalogue number 39335) and beta-tubulin (Cell Signalling Technology, catalogue number 21465) and then with secondary anti-rabbit IgG labelled with horseradish peroxidase (Cell Signalling Technology, catalogue number 70745). Finally, an electrochemiluminescence (ECL™ Western Blotting Reagents, GE Healthcare) system was used to detect proteins.

2.12 Raman spectroscopy measurements and analyses

For Raman spectroscopy measurements, hMSC were cultured as described in section 2.2 but were seeded on MgF₂ coverslips (Crystan, UK) inside a petri dish to facilitate Raman spectral analyses, as MgF₂ is a weak Raman scatterer, whilst tissue culture plastic produces an intense Raman signal (Notingher et al., 2004). After 21 days in culture, MgF₂ coverslips were briefly rinsed with dH₂O and dried in a bell jar desiccator (Gentleman et al., 2009). Raman measurements were performed using a custom-built Raman system (University of Southern Denmark, Odense) consisting of a Laser Quantum Ventus 532 nm Laser (Stockport, UK). The laser was coupled via free space optics into an Olympus BX60 microscope (Hamburg, DE). Raman scattered light was collected through a 50x /NA 0.8 objective and fibre coupled into an Acton SpectraPro 2500i f/6.5 spectrograph, using a 1200lines/mm grating with a

Princeton Instruments PIXIS 400F 1340 x 400 pixel CCD camera (Trenton, NJ) operating at -75°C. Integration time was 10 seconds, averaged 3 times for each spectrum, using 30 mW at the sample. It had previously been shown that these conditions do not cause spectral changes due to sample heating/burning (Evans et al., 2012). For univariate peak analyses, between 295 and 315 spectra were examined per group.

Data were smoothed using a 5-point Savitzky-Golay filter and background corrected by a 5th order polynomial using an automated weighted least squares fitting method (Lieber and Mahadevan-Jansen, 2003). To determine the position of the $PO_4^{3-} \nu_1$ peak at $\sim 960\text{ cm}^{-1}$, a single Gaussian was fit to the data, as previously described (Morris and Mandair, 2010, Wallace et al., 2009). Gaussian fits were used to calculate the $\sim 960\text{ cm}^{-1}$ ($PO_4^{3-} \nu_1$) and 1660 cm^{-1} (Amide I) peak areas. The mineral to matrix ratio was calculated by determining the ratio of the area of the 960 cm^{-1} peak to that of the 1660 cm^{-1} peak (Gentleman et al., 2009, Tarnowski et al., 2003) after normalization using Multiplicative Scatter Correction. Spectra with a $PO_4^{3-} \nu_1$ peak area of less than 500 were excluded from multivariate analyses and in total, spectra collected from 29 control-, 38 DBEq- and 42-bortezomib treated cultures were analysed. For classification analyses, 6-component Partial Least Squares-Discriminant Analysis model was fitted and cross validated using venetian blinds and 10 splits.

2.13 Atomic force microscopy (AFM) measurements of nodule stiffness and adhesion

Glass coverslips measuring 22 mm were prepared by soaking in FBS overnight and allowing to air dry. hMSC were seeded at 190,000 cells/coverslip. The next day, basal medium was replaced with osteogenic medium and cells were differentiated as described above. After 21 days, coverslips were frozen slowly (Gentleman et al., 2009). Briefly, medium was aspirated from plates and replaced with a 1:1 solution of 20% DMSO in FBS:basal medium. Plates were then sealed with parafilm and placed within 2 nesting polystyrene boxes at -80°C. Samples were defrosted at room temperature immediately prior to measurements. Coverslips were immobilised on 60 mm diameter tissue culture dishes (TPP, CH) by gluing small coverslips to the plate at the sample edges. Coverslips were immersed in PBS (without calcium and magnesium, pH 7.4, Gibco) and measurements were made at room temperature. Mineralised nodules were identified from bright field images as dense, opaque patches on the culture surface that varied in diameter between ~35 and 125 μm . Indentation measurements were carried out on a JPK Nanowizard® I AFM equipped with a cantilever (cantilever B, spring constant, $K \sim 16 \text{ N/m}$) with a pyramidal, silicon AFM probe (HQ:NSC35/Hard/AL BS, MikroMasch®, DE). Indentations were carried out at 1 Hz under a constant loading rate with a relative setpoint force set as 3650 nN and no dwelling time. The effect of the hard underlying substrate was assumed as negligible as the indenter penetration was less than 10% of the nodule thickness (Jung et al., 2004, Sirghi et al., 2008). Nanoindentations were made at between 1 and 5 locations on each of the nodule's surface. 20 individual

force indentation curves were made at each location ($1 \times 1 \mu\text{m}^2$). Measurements were performed on at least 12 nodules per donor per treatment. Young's modulus (E), maximum adhesion force, maximum length of adhesion interactions, and adhesion energy were determined in JPK SPM software using the Hertz model. The Poisson's ratio of both the sample and tip were assumed to be 0.3 (Lefèvre et al., 2013).

2.14 Transmission electron microscope (TEM) imaging

Differentiated hMSC cultures were scraped from plates and pelleted in PBS. Pellets were fixed with 1% (w/v) glutaraldehyde for 15 minutes, then washed in cacodylate buffer three times, followed by incubation in 1% (w/v) osmium tetroxide and 1.5% (w/v) potassium ferrocyanide in H_2O for 1 hour. Pellets were then washed in H_2O three times and dehydrated in an ethanol/distilled water series at concentrations from 20 to 100% ethanol for 15 minutes in each. Samples were then infiltrated with Epon 812 (EMS) epoxy resin in absolute ethanol at ratios of 3:1, 2:1, 1:1, 1:2, 1:3 (ethanol:resin) and then 100% epoxy resin for 6 hours each. Samples were then cured at 60°C for 48 hours. Resin blocks were sectioned on a Leica Ultracut EM FC7 ultramicrotome on a ultracut UC7 chassis into 80 nm sections, placed on grids, stained with Reynold's lead citrate for 15 minutes and 1% (w/v) uranyl acetate for 15 minutes, then imaged on a Jeol 1010 TEM at 80 KV.

2.15 Scanning electron microscopy (SEM) imaging

Cultures in well plates were dehydrated using increasing concentrations of ethanol (20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 100%, and 100% for 10 minutes each) and mounted on SEM stubs using carbon tape. Silver painting and carbon coating was carried out using a Quorum K975X Carbon coater. Samples were imaged on either a Hitachi S-3499N or a Zeiss Sigma. For DDC-SEM images, secondary electron (SE) mode was used to obtain topographic information, whilst the backscattered electron detector (BSE) was applied to differentiate between organic and inorganic material. DDC-SEM images were produced by assigning different colours to the BSE and SE modes using Image J.

2.16 Statistical analyses

Data presented are medians of 3 independent experiments for cell biology experiments, each performed in triplicate, unless stated otherwise. Statistical analyses for all measurements were carried out using a non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test unless stated otherwise. All analyses were carried out using GraphPad Prism version 7 for Windows (GraphPad Software, USA). Significant differences in the distributions of adhesion values between treatments were evaluated using Matel-Haenszel linear-by-linear association Chi-squared (χ^2) test for trend. Power was evaluated by determining Goodman and Kruskal's gamma (γ) and standardised residuals (SR) were used to identify the most significant intervals that contributed to differences between

histograms, all three using IBM® SPSS® statistics version V23. p values are indicated in figure captions, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

3 Results

To understand how impaired proteostatic fine-tuning functionally affected tissue, I created an in vitro model using intermittent low-level proteasome or VCP/p97 inhibition in hMSC as they differentiated into osteoblasts and formed cell-derived, bone-like material. Figure 10 shows the experimental approach used in this project.

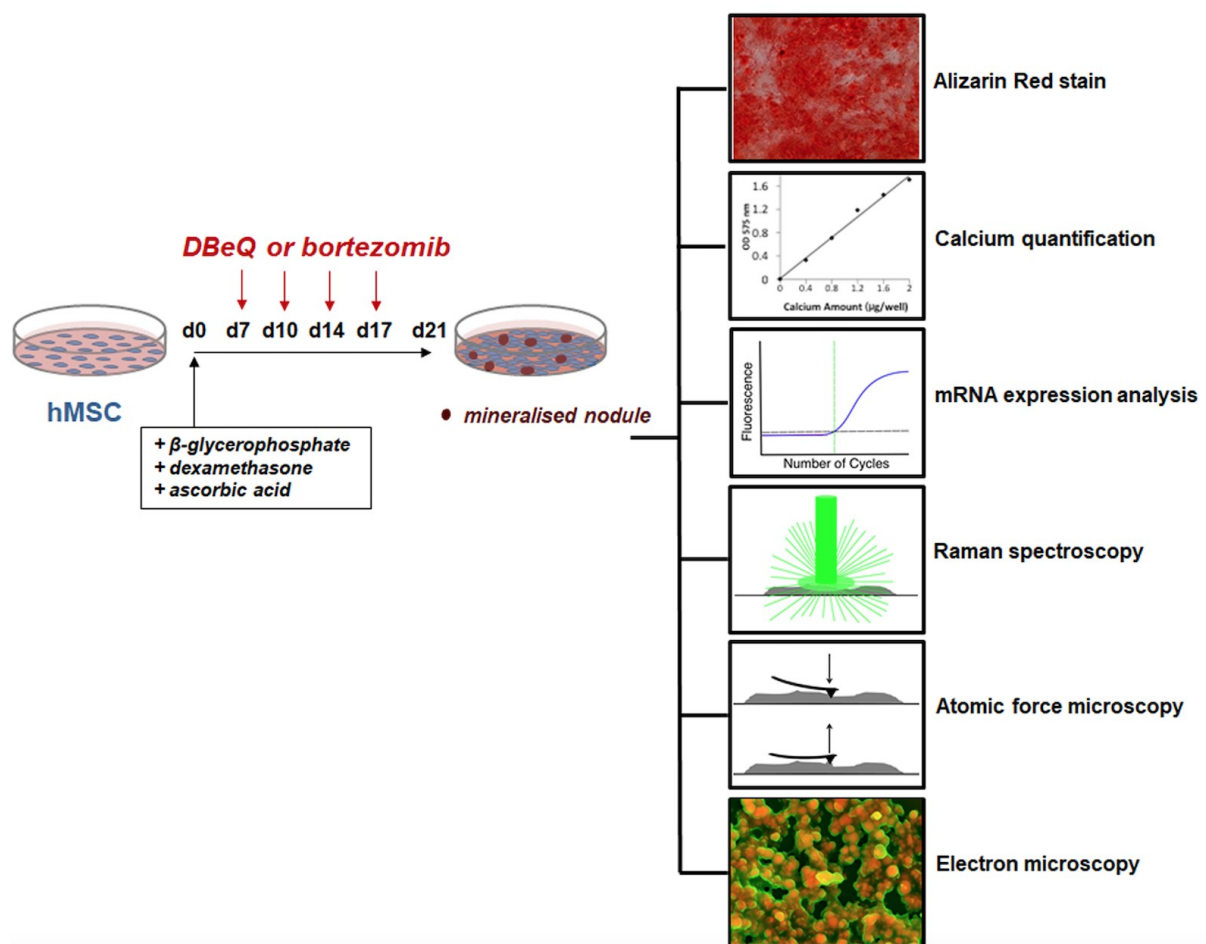


Figure 10. Schematic describing the experimental approach. hMSC were induced to undergo osteogenic differentiation in vitro, and DBeQ or bortezomib were added on days 7, 10, 14 and 17 to inhibit VCP/p97 or the proteasome. After 21 days, mineralised cultures were analysed using an array of biological and physical science characterisation techniques.

3.1. Commercial and classical 'in-house' prepared media result in similar levels of osteogenic differentiation

I first set out to test whether classical in-house prepared osteogenic medium (containing ascorbic acid, β -glycerophosphate, and dexamethasone) and the commercially available Stembro® Osteogenesis Differentiation kit would result in a similar degree of differentiation of hMSCs. This was assessed by comparing mineralisation and calcium content after 21 days of culture. Figure 11a shows that mineralised nodule formation, Figure 11b that quantification of mineralisation and Figure 11c that calcium content were similar in cells differentiated with Stembro® and classical medium with no significant difference seen between the two media (Table 1). Next, osteogenic differentiation of hMSCs in classical medium was further tested by measuring alkaline phosphatase (ALP) activity. As shown in Figure 11d, ALP activity, a biochemical osteoblast marker (Abildgaard et al., 2000, Christenson, 1997, Rutkovskiy et al., 2016), increased with the duration of osteogenic differentiation.

These results indicated that classical in-house prepared medium resulted in comparable degrees of osteogenic differentiation as a commercially available product. Considering the high cost and non-disclosed composition of the commercial medium, I decided to use the in-house made classical osteogenic medium for further experiments.

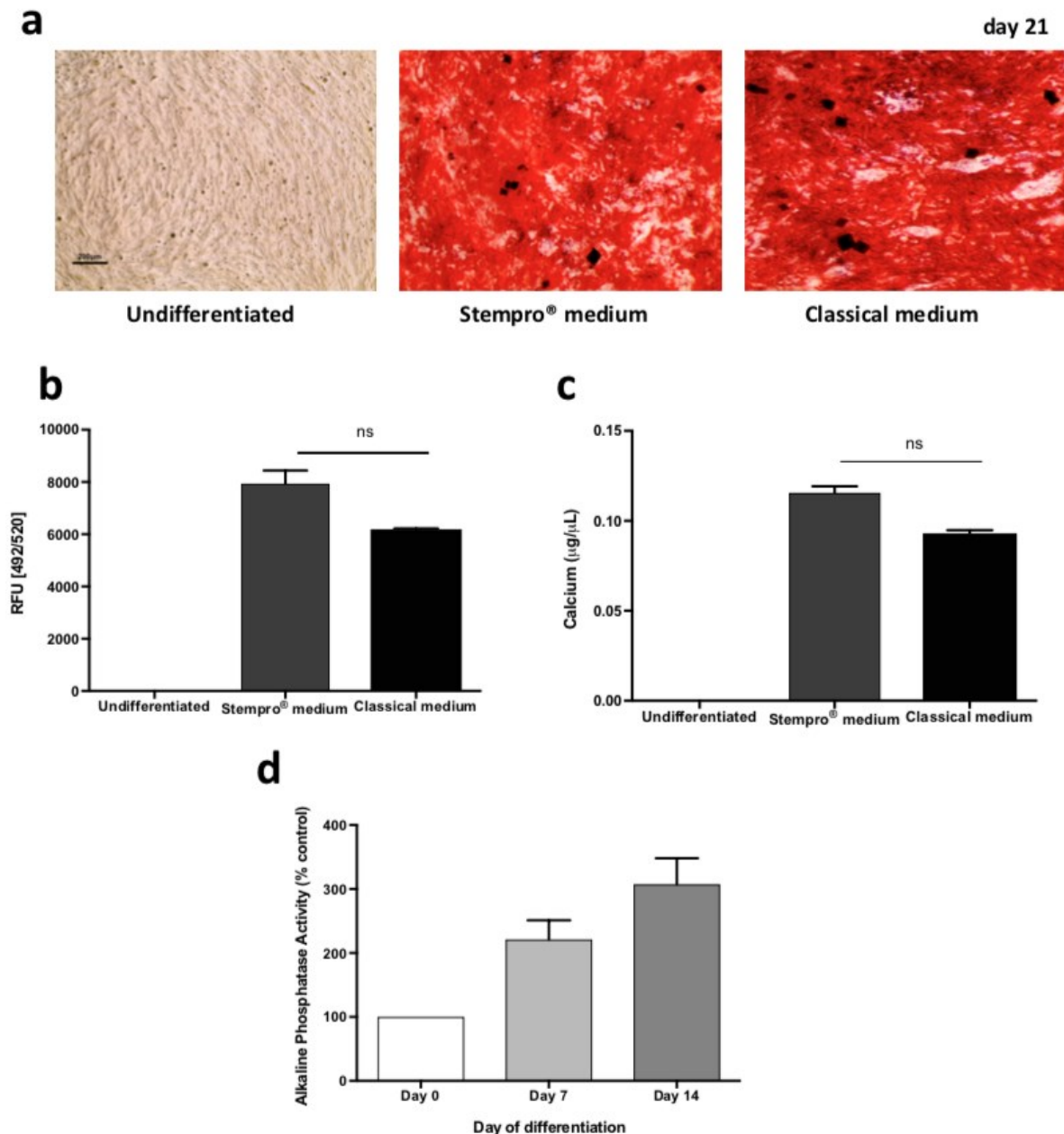


Figure 11. Commercial and classical ‘in-house’ prepared media result in similar levels of osteogenic differentiation. (a) Micrographs showing lack of mineralisation as determined by Alizarin Red staining in hMSCs maintained in non-differentiating medium (left), and similar amounts of mineralisation and mineralised nodule formation in cells differentiated for 21 days in commercially available Stempro® medium or classical osteogenic medium (scale bar = 200µm). (b) Mineralisation quantification of differentiated hMSC and (c) colorimetric calcium content quantification showing no significant difference between Stempro® medium or classical osteogenic medium after 21 days of culture. (d) Increasing alkaline phosphatase activity in hMSCs undergoing differentiation with classical osteogenic medium for the indicated number of days. Plots show mean + SEM ($n=3$).

Table 1. Statistical analyses of mineralisation quantification and colorimetric calcium content quantification in hMSC differentiated in commercially available Stempro® medium or classical osteogenic medium after 21 days of culture.

Mineralisation quantification	
Stempro® vs. Classical	$p = 0.5391$
Colorimetric calcium content quantification	
Stempro® vs. Classical	$p = 0.5421$

Statistical analyses were carried out using a non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test. $n = 3$, from 3 different donors.

3.2 DBeQ and bortezomib induce a mild proteotoxic stress response in differentiating hMSC

To develop an *in vitro* model of proteostasis imbalance, I first aimed to determine if I could mildly perturb proteostasis in hMSC. Genetic approaches to deplete VCP/p97 or the proteasome are not suitable to study the effects of mild functional impairments as knockdown or knockout approaches cannot be finetuned and would result in considerable functional loss (Anderson et al., 2015, Muller et al., 2007). Therefore, I took a pharmacological approach and treated hMSC with either the well-characterised and highly selective VCP/p97 inhibitor, DBeQ (Bastola et al., 2016, Chou et al., 2011a, Chou and Deshaies, 2011, Parzych et al., 2015a), or the first-in-class clinical proteasome inhibitor, bortezomib (Richardson and Anderson, 2003). While it cannot be completely excluded that both inhibitors have off-target effects, the low concentrations used in my experiments makes them very unlikely.

3.2.1 hMSC and osteosarcoma cells are resistant to proteasome inhibition but sensitive to VCP/p97 inhibition

To evaluate the effects of VCP/p97 and proteasome inhibitors on the viability of hMSC, I measured the metabolic activity of hMSC after treatment with bortezomib or DBeQ using the Alamar Blue assay which measures overall metabolic activity. Figure 12a shows that undifferentiated hMSC from two different donors were found to be highly insensitive to proteasome inhibition, their viability largely unaffected by bortezomib even after treatment with very high concentrations of inhibitor.

Similarly, Saos-2 osteosarcoma cells were insensitive to proteasome inhibition with bortezomib (Figure 12c). I decided to assess the effect of bortezomib on OPM-2 multiple myeloma cells known to be sensitive to proteasome inhibition by bortezomib (Auner et al., 2013). As expected, OPM-2 cells were sensitive to bortezomib, which induced cell death at an IC_{50} of 7.5 nM (Figure 13 and Table 2). In contrast to proteasome inhibition with bortezomib, Figure 12b demonstrates that undifferentiated hMSC were susceptible to VCP/p97 inhibition with DBeQ, which induced cell death with an IC_{50} of 7.84 μ M (donor 1) and 6.95 μ M (donor 2) (Table 2). Saos-2 cells were also sensitive to p97 inhibition with DBeQ, inducing cell death with an IC_{50} of 12.95 μ M (Figure 12d and Table 2).

Thus, undifferentiated hMSC and osteosarcoma cells do not undergo cell death in response to proteasome inhibition but die following disruption of VCP/p97 function.

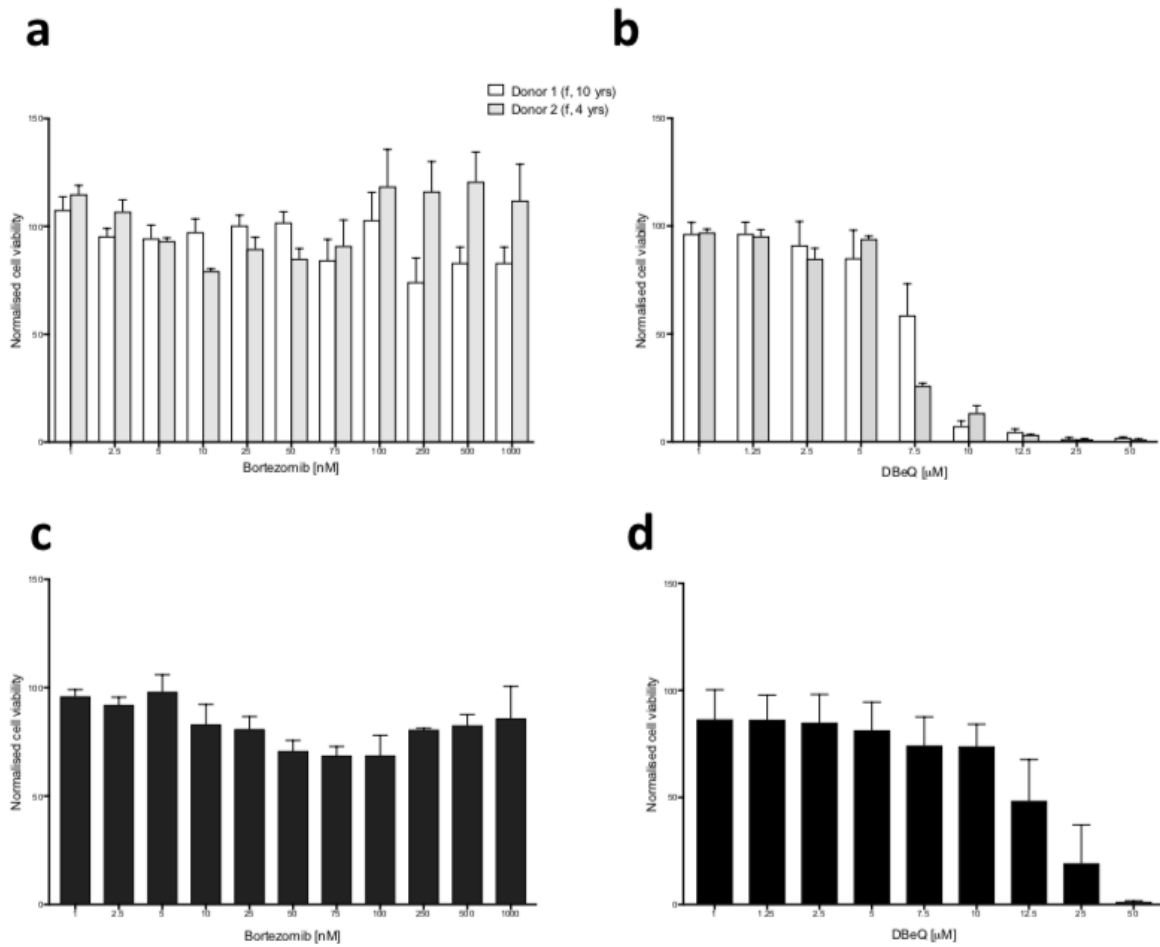
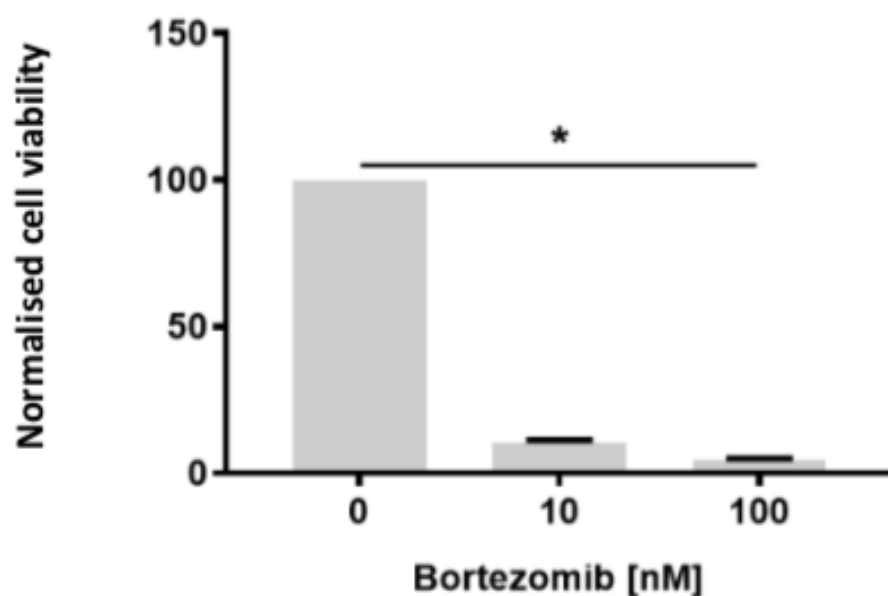


Figure 12. hMSC and osteosarcoma cells are resistant to proteasome inhibition but sensitive to VCP/p97 inhibition. Normalised viability of hMSC from two different donors treated with the indicated concentrations of (a) bortezomib and (b) DBeQ for 24 h. Normalised viability of Saos-2 cells treated with (c) bortezomib, and (d) DBeQ for 24 h. Plots show mean + SEM ($n=3$).



control vs. bortezomib 10 nM	$p = 0.517$
control vs. bortezomib 100 nM	$p = 0.019$
bortezomib 10 nM vs. 100 nM	$p = 0.517$

Figure 13. OPM-2 cells are sensitive to proteasome inhibition. Normalised cell viability of OPM-2 cells treated for 48 h with bortezomib (10 nM and 100 nM) or vehicle control. Plots show mean + SEM ($n=3$). Statistical analyses were carried out using a non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test and the detailed p values are indicated in the table.

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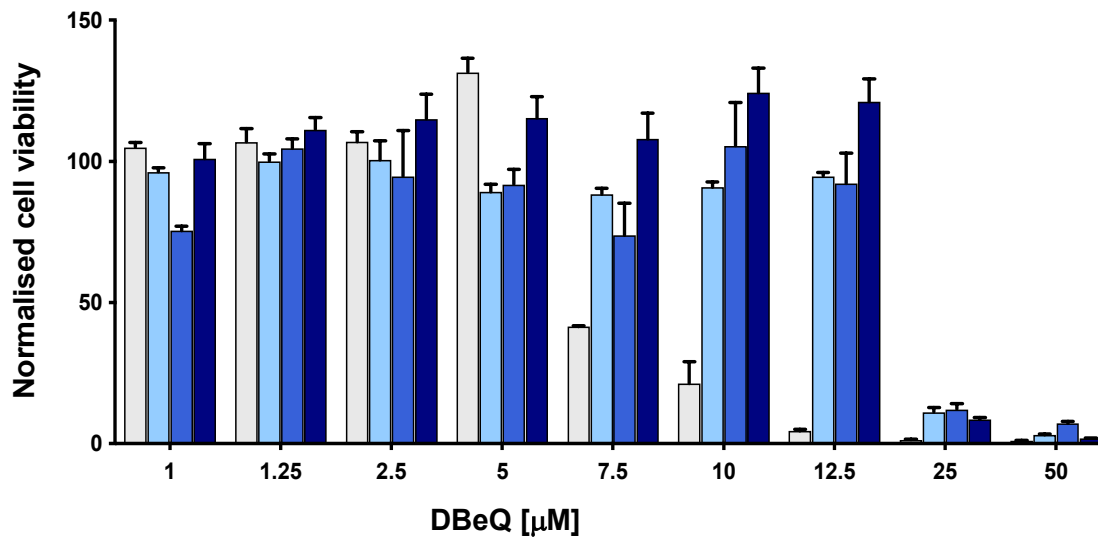
Table 2. IC₅₀ values of different cell types after treatment with DBeQ or bortezomib for 24 h (*n*=3).

Cell type	Inhibitor	IC ₅₀
hMSC Donor 1	DBeQ	7.84 µM
hMSC Donor 2	DBeQ	6.95 µM
Saos-2	DBeQ	12.95 µM
OPM-2	Bortezmib	7.5 nM

3.2.2 Osteogenic differentiation decreases the sensitivity of hMSC to VCP/p97 inhibition

Next, I aimed to determine if I could mildly perturb proteostasis in hMSC undergoing osteogenic differentiation. To define inhibitor concentrations that would induce mild functional impairment without overt toxic effects, I determined IC₅₀ values for viability during the 21-day differentiation process. Figure 14a demonstrates that osteogenic differentiation increased the IC₅₀ for DBeQ as determined by cellular metabolic activity from 7.4 μ M in undifferentiated hMSC to 23 μ M in their differentiated progeny. These data suggest that differentiation decreases the dependence of hMSC on functional VCP/p97. Bortezomib, did not reduce viability of differentiating hMSC at concentrations up to 1000 nM at any point during the differentiation process (Figure 14b).

a



day	IC ₅₀
0	7.4
7	19.9
14	23.6
21	23.1

b

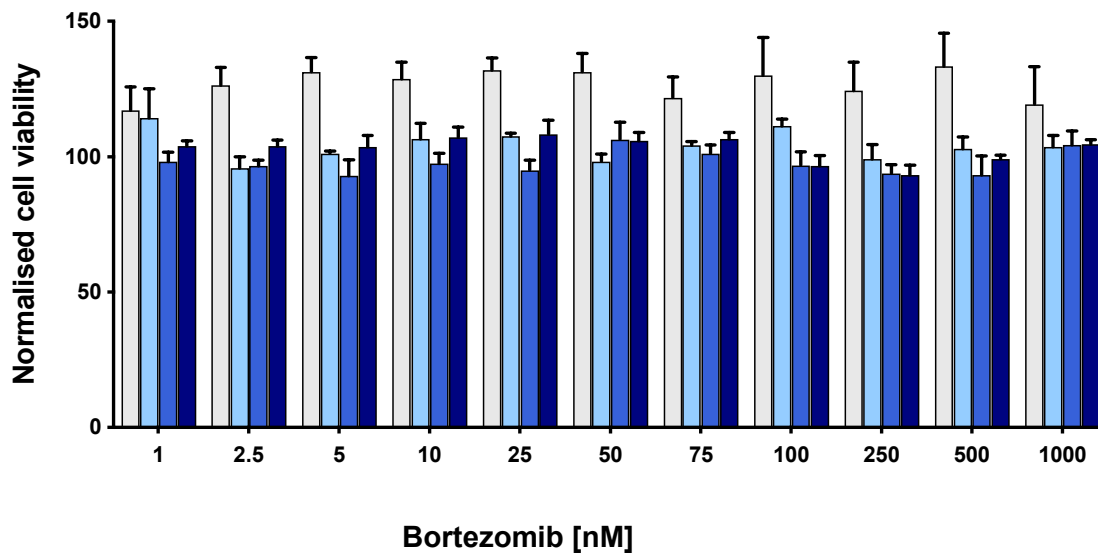


Figure 14. Osteogenic differentiation decreases the sensitivity of hMSC to p97 inhibition. Normalised viability of hMSC undergoing osteogenic differentiation treated on days 0, 7, 14, and 21 with the indicated concentrations of (a) DBeQ or (b) bortezomib for 48 h. Plots show mean + SEM ($n=3$). For detailed n and p values see Supplementary Table 3 and 4. Reproduced from (Loaiza et al., 2018) under the terms of the Creative Commons Attribution Non-Commercial 4.0 International Public License.

3.2.3 Inhibition of the proteasome and VCP/p97 results in different transcriptional responses in hMSC and osteosarcoma cells

To gain further insight into the proteotoxic stress caused by VCP/p97 and proteasome inhibition, I set out to analyse the expression of a panel of genes encoding proteins with key roles in proteostasis after treating Saos-2 cells and undifferentiated hMSC with different concentrations of DbeQ and a clinically relevant concentration of bortezomib. Furthermore, I used the classical proteotoxic stress inducer tunicamycin, known to cause protein misfolding in the endoplasmic reticulum by blocking protein glycosylation (Guha et al., 2017), as a control.

I chose to analyse the expression of *HSPA5* (heat shock protein family A member 5; alias *BIP* (immunoglobulin heavy-chain-binding protein)) and *DNAJC3* (DNA J heat shock protein family (Hsp40) member C3; aliases *P58* and *P58IPK*) as examples of endoplasmic reticulum (ER) chaperones which are expected to be upregulated in response to misfolded proteins. To study the PERK pathway, which is also activated during ER stress, I analysed the expression of *ATF4* (activating transcription factor 4), *DDIT3* (DNA damage inducible transcript 3; alias *CHOP* (transcription factor C/EBP homologous protein)) and *PPP1R15A* (protein phosphatase 1 regulatory subunit 15A; alias *GADD34* (growth arrest and DNA damage-inducible 34)) which form a key regulatory feedback loop that fine-tunes protein translation as part of the UPR. I also analysed expression of *NFE2L1* (nuclear factor, erythroid 2 like 1; alias *TCF11* (transcription factor 11)), a transcription factor involved in the regulation of proteasome components upon proteasome inhibition, *TXNIP* (thioredoxin interacting protein), a mediator involved in switching the adaptive

UPR to apoptotic ER stress signaling, and *VCP/p97* (Anthony and Wek, 2012, Osowski et al., 2012, Steffen et al., 2010).

DBeQ and bortezomib induced a very mild early transcriptional proteotoxic stress response in Saos-2 cells, as determined by low-level changes in proteostasis gene mRNA levels (Figure 15). At 16h, changes were more pronounced. *CHOP*, *GADD34*, and *BIP* were up-regulated in a concentration-dependent manner by VCP/P97 inhibition (Figure 16). DBeQ also induced the expression of *P58* and *TCF11* and down-regulated, in a concentration-dependent manner, the expression of *TXNIP* (Figure 16). In comparison, the effect of bortezomib on mRNA expression levels was very limited (Figure 16).

I then asked if the response observed in osteosarcoma cells was comparable in hMSC. As most changes observed in Saos-2 cells were seen after late proteostasis perturbation, the 16 h time-point was selected to study the transcriptional response in hMSC to VCP/P97 and proteasome inhibition. Treatment with the highest concentration of DBeQ (15 μ M) was omitted from this set of experiments due to the effect on cellular viability as illustrated by the lower IC₅₀ in hMSC when compared to Saos-2 cells. DBeQ induced a mild up-regulation of *CHOP*, *GADD34*, *P58*, *VCP/p97* and *TCF11* and a concentration-dependent decrease in expression of *TXNIP* in hMSC (Figure 17). In contrast, the effect of bortezomib on mRNA expression levels was not significant. Tunicamycin, however, strongly upregulated most of the mRNAs tested (Figure 17). Thus, bortezomib and DBeQ have different effects on the transcription of selected genes of the secretory network in undifferentiated hMSC and Saos-2 cells. These effects also differ from those induced by tunicamycin.

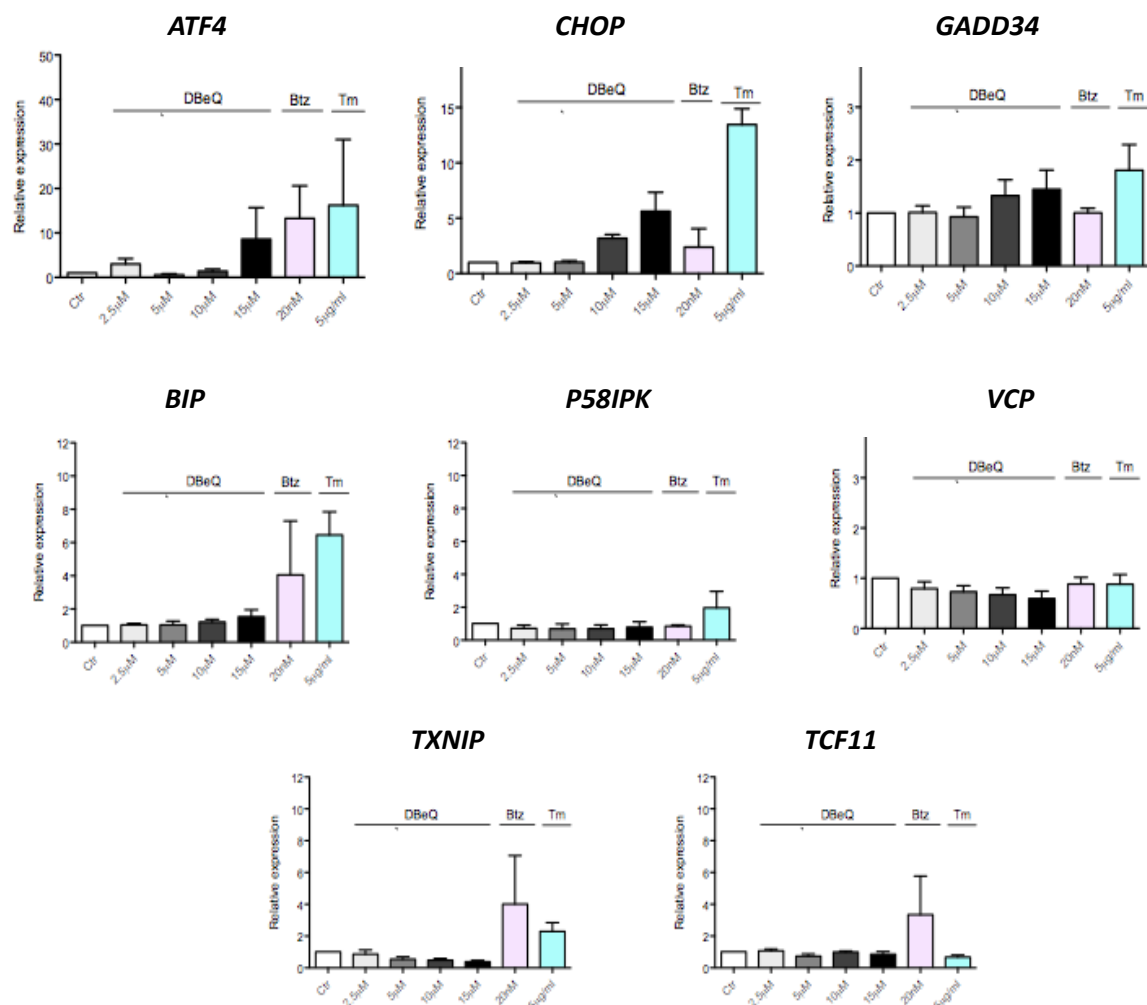


Figure 15. Early inhibition of the proteasome and VCP/p97 results in different transcriptional responses in osteosarcoma cells. Relative mRNA expression levels (normalised to untreated controls) for *ATF4*, *CHOP*, *GADD34*, *BIP*, *P58IPK*, *VCP*, *TXNIP* and *TCF11* in Saos-2 cells after treatment for 2 h with DBeQ (2.5 μ M, 5 μ M, 10 μ M, 15 μ M), bortezomib (20 nM), or tunicamycin (5 μ g/mL). Plots show mean + SEM ($n=3$).

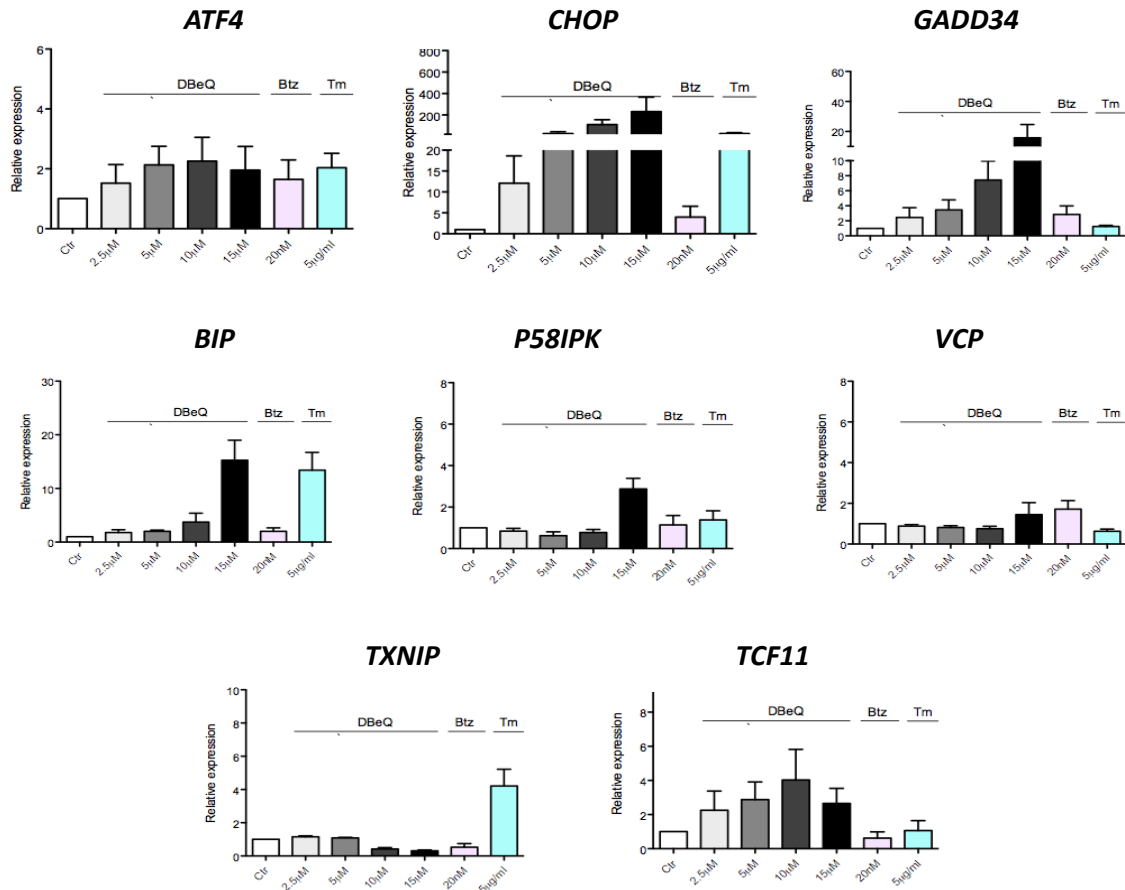


Figure 16. Late inhibition of the proteasome and VCP/p97 results in different transcriptional responses in osteosarcoma cells. Relative mRNA expression levels (normalised to untreated controls) for *ATF4*, *CHOP*, *GADD34*, *BIP*, *P58IPK*, *VCP*, *TXNIP* and *TCF11* in Saos-2 cells after treatment for 16 h with DBeQ (2.5 μ M, 5 μ M, 10 μ M, 15 μ M), bortezomib (20 nM), or tunicamycin (5 μ g/mL). Plots show mean + SEM (n=3).

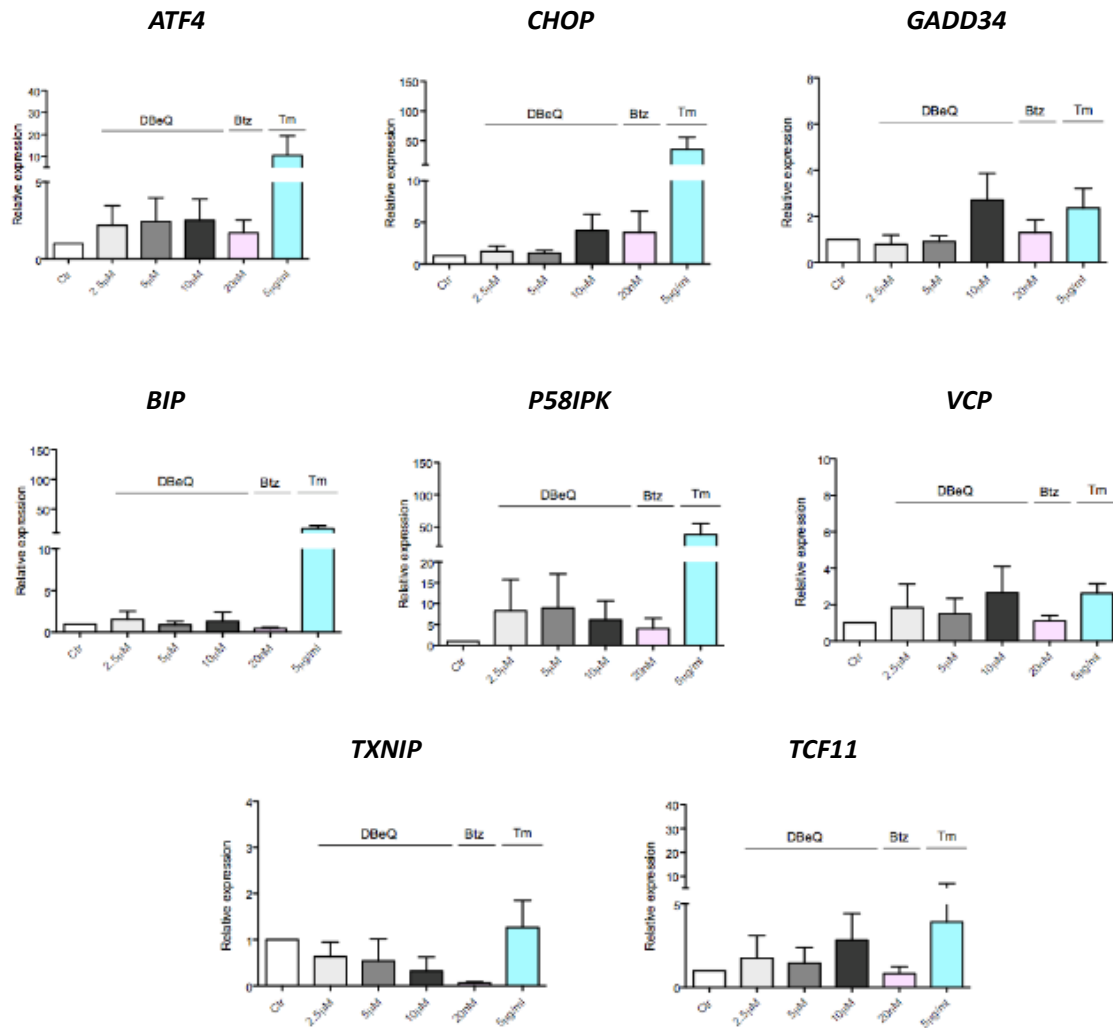


Figure 17. Late inhibition of the proteasome and VCP/p97 results in different transcriptional responses in hMSC. Relative mRNA expression levels (normalised to untreated controls) for *ATF4*, *CHOP*, *GADD34*, *BIP*, *P58IPK*, *VCP*, *TXNIP* and *TCF11* in hMSC after treatment for 16 h with DBeQ (2.5 μ M, 5 μ M, 10 μ M), bortezomib (20 nM), or tunicamycin (5 μ g/mL). Plots show mean + SEM ($n=3$).

3.2.4 DBeQ and bortezomib induce a mild proteotoxic stress response in differentiating hMSC

Next, I aimed to determine the degree of proteotoxic stress caused by a concentration of DBeQ that did not affect viability (5 μ M) during *in vitro* differentiation compared to a clinically relevant concentration of bortezomib (20 nM) by quantifying the expression of the panel of genes encoding proteins with key roles in proteostasis described in 3.2.3. DBeQ and bortezomib both induced a very mild proteotoxic stress response, as determined by low-level changes in proteostasis mRNA levels that were largely non-significant (Figure 18 and Table 3). For comparison, tunicamycin, resulted in more pronounced changes in proteostasis gene mRNAs when given at a nonlethal concentration (Figure 18 and Table 3). Importantly, immunoblotting for ubiquitinated proteins in cells treated with bortezomib and DBeQ demonstrated a clear increase in the level of ubiquitinated proteins in cells treated with bortezomib, confirming effective proteasome inhibition, while DBeQ had a minor effect (Figure 19). These observations demonstrate that by fine-tuning an appropriate dose of DBeQ and bortezomib in differentiating hMSC, it is possible to impair the UPS and trigger mild proteotoxic stress that is not acutely toxic to cells, thereby mimicking chronic proteostatic imbalances.

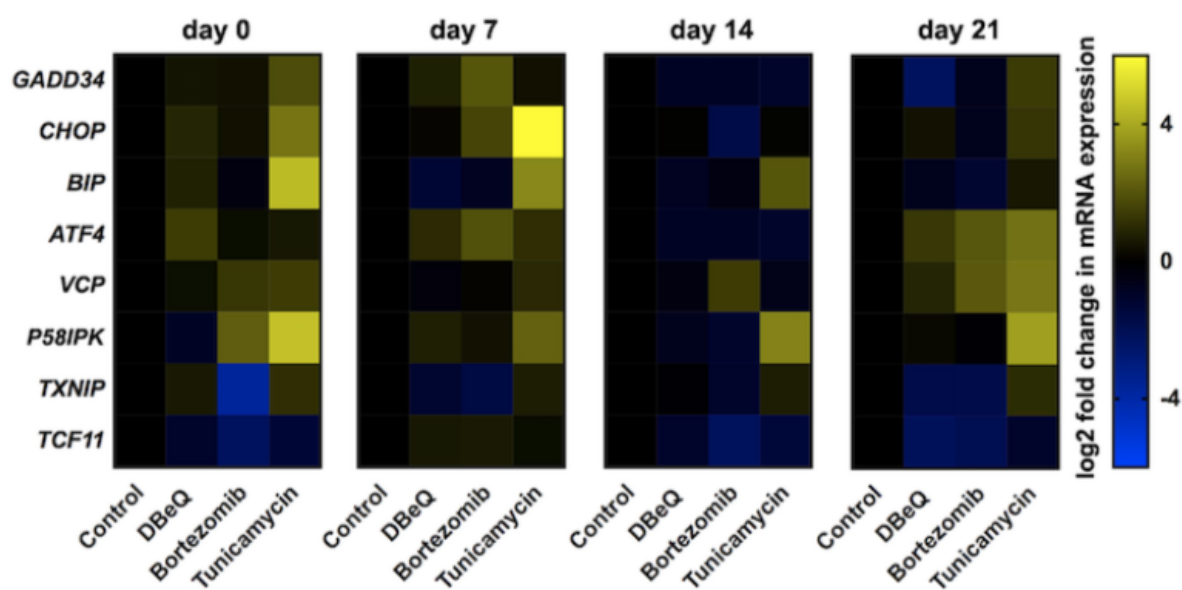


Figure 18. DBeQ and bortezomib induce a mild proteotoxic stress response in differentiating hMSC. Heatmaps showing relative mRNA expression levels (normalised to undifferentiated controls) for *GADD34*, *CHOP*, *BIP*, *ATF4*, *VCP*, *P58IPK*, *TXNIP* and *TCF11* in hMSC treated on days 0, 7, 14 or 21 of osteogenic differentiation with DBeQ (5 μ M), bortezomib (20 nM), or tunicamycin (5 μ g/mL). Heatmaps show mean of log 2-fold changes in gene expression (normalised to undifferentiated controls) for hMSC from 3 different donors.

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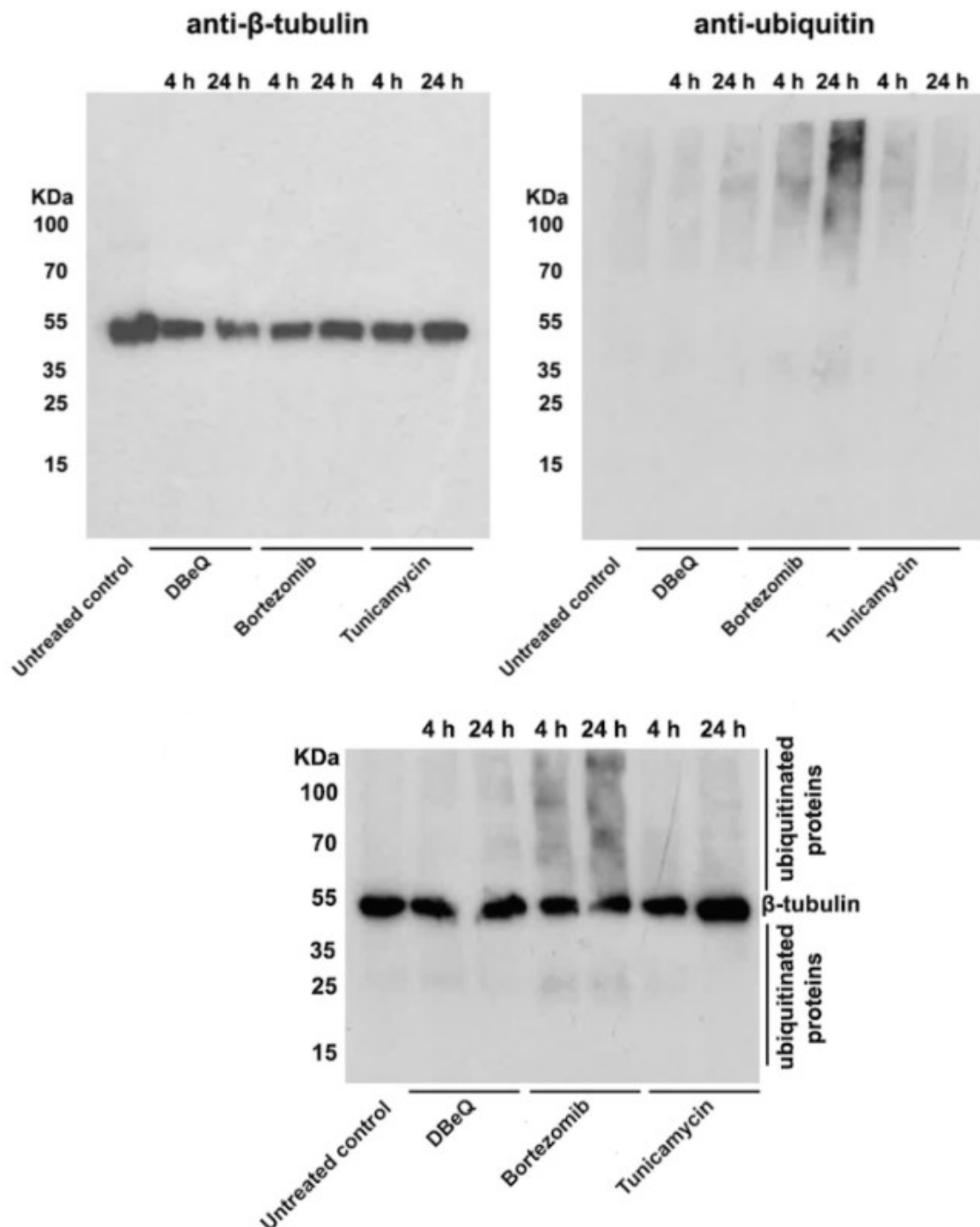


Figure 19. Proteasome and VCP/p97 inhibition impair the clearance of ubiquitinated proteins in hMSC. Immunoblotting for β -tubulin and ubiquitinated proteins alone or combined (independent experiment) on whole cell extracts from undifferentiated hMSC untreated (control) or treated for 4 h or 24 h with DBeQ at 5 μ M, bortezomib at 20 nM, or tunicamycin at 5 μ g/mL.

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Table 3. mRNA expression analysis of hMSC (n = 3) treated on days 0, 7, 14, or 21 of osteogenic differentiation with DBeQ (5 μ M), bortezomib (20 nM), or tunicamycin (5 μ g/mL) for 24 h. Results shown are log₂ fold changes in mRNA expression compared

to vehicle-treated controls and correspond to those shown in Figure 3.2.4a heatmaps. Statistical analyses were carried out using a two-way ANOVA and Dunnett's multiple comparisons test. Significant results (p<0.05) are highlighted in red.

	DBeQ				bortezomib				tunicamycin			
	mean	$\pm$	SEM	p	mean	$\pm$	SEM	p	mean	$\pm$	SEM	p
day 0												
<i>GADD34</i>	0.534	$\pm$	0.356	0.937	0.535	$\pm$	0.369	0.937	1.409	$\pm$	1.101	0.469
<i>CHOP</i>	0.273	$\pm$	0.631	0.991	-0.090	$\pm$	0.785	1.000	3.249	$\pm$	1.344	0.016
<i>BIP</i>	-0.229	$\pm$	1.105	0.994	-0.248	$\pm$	0.165	0.993	4.622	$\pm$	0.800	0.000
<i>ATF4</i>	-0.169	$\pm$	1.632	0.998	-0.037	$\pm$	0.649	1.000	1.424	$\pm$	0.892	0.460
<i>VCP</i>	0.097	$\pm$	0.237	1.000	1.280	$\pm$	0.216	0.543	1.250	$\pm$	0.555	0.561
<i>P58IPK</i>	-0.196	$\pm$	0.791	0.997	1.776	$\pm$	0.733	0.286	4.015	$\pm$	1.625	0.002
<i>TXNIP</i>	-0.737	$\pm$	1.524	0.856	-2.822	$\pm$	0.947	0.043	0.844	$\pm$	1.152	0.801
<i>TCF11</i>	-1.148	$\pm$	0.855	0.623	-2.118	$\pm$	0.131	0.166	-0.816	$\pm$	1.126	0.816
day 7												
<i>GADD34</i>	0.161	$\pm$	1.076	0.999	1.724	$\pm$	0.375	0.635	1.012	$\pm$	0.881	0.889
<i>CHOP</i>	1.261	$\pm$	1.339	0.811	3.125	$\pm$	1.879	0.187	5.902	$\pm$	1.849	0.004
<i>BIP</i>	-1.319	$\pm$	1.309	0.791	-0.325	$\pm$	1.076	0.995	3.722	$\pm$	0.903	0.093
<i>ATF4</i>	0.952	$\pm$	1.648	0.905	2.560	$\pm$	1.210	0.330	2.374	$\pm$	1.559	0.390
<i>VCP</i>	-0.172	$\pm$	0.231	0.999	0.778	$\pm$	0.820	0.945	0.925	$\pm$	0.843	0.912
<i>P58IPK</i>	1.353	$\pm$	1.224	0.778	1.350	$\pm$	2.336	0.780	3.455	$\pm$	2.164	0.129
<i>TXNIP</i>	-1.323	$\pm$	0.450	0.789	-1.090	$\pm$	0.578	0.867	0.640	$\pm$	0.892	0.976
<i>TCF11</i>	-1.750	$\pm$	2.778	0.624	-0.252	$\pm$	1.161	0.998	-1.090	$\pm$	1.951	0.867
day 14												
<i>GADD34</i>	-2.036	$\pm$	1.180	0.635	-1.275	$\pm$	2.232	0.870	-1.706	$\pm$	1.434	0.745
<i>CHOP</i>	-2.569	$\pm$	3.781	0.460	-2.323	$\pm$	3.612	0.539	-0.743	$\pm$	1.876	0.969
<i>BIP</i>	-0.679	$\pm$	0.243	0.976	-1.152	$\pm$	0.871	0.899	2.872	$\pm$	1.000	0.371
<i>ATF4</i>	-2.036	$\pm$	1.180	0.635	-1.275	$\pm$	2.232	0.870	-1.706	$\pm$	1.434	0.745
<i>VCP</i>	-0.279	$\pm$	1.021	0.998	1.239	$\pm$	1.162	0.879	0.508	$\pm$	1.141	0.990
<i>P58IPK</i>	-0.749	$\pm$	0.620	0.968	-0.001	$\pm$	1.939	1.000	2.289	$\pm$	0.894	0.550
<i>TXNIP</i>	0.463	$\pm$	1.287	0.992	-0.330	$\pm$	0.894	0.997	1.633	$\pm$	1.239	0.767
<i>TCF11</i>	-0.799	$\pm$	1.397	0.962	-1.363	$\pm$	1.125	0.847	-1.112	$\pm$	1.575	0.908
day 21												
<i>GADD34</i>	-1.980	$\pm$	1.163	0.369	-0.174	$\pm$	0.969	0.999	0.942	$\pm$	1.006	0.845
<i>CHOP</i>	-0.503	$\pm$	0.998	0.971	-0.846	$\pm$	0.532	0.881	1.137	$\pm$	1.034	0.761
<i>BIP</i>	-0.367	$\pm$	0.565	0.988	-1.166	$\pm$	0.685	0.748	1.273	$\pm$	2.425	0.697
<i>ATF4</i>	1.582	$\pm$	0.438	0.546	1.168	$\pm$	1.026	0.747	-0.262	$\pm$	3.281	0.996
<i>VCP</i>	0.779	$\pm$	0.392	0.904	1.856	$\pm$	0.259	0.420	2.689	$\pm$	0.386	0.152
<i>P58IPK</i>	0.181	$\pm$	1.056	0.999	0.509	$\pm$	1.130	0.970	3.348	$\pm$	0.852	0.055
<i>TXNIP</i>	-0.692	$\pm$	1.220	0.929	-1.681	$\pm$	0.222	0.500	1.266	$\pm$	0.918	0.701
<i>TCF11</i>	-1.379	$\pm$	1.001	0.645	-1.869	$\pm$	0.287	0.415	-0.678	$\pm$	0.897	0.933

3.3 Neither VCP/p97 nor proteasome inhibition affect gross measures of hMSC osteogenic differentiation

Like primary osteoblasts, hMSC form bone-like mineralised nodules *in vitro* in response to chemical induction. Conventional methods to evaluate bone nodule formation and calcification are well established and commonly used (Evans et al., 2012, Hanna et al., 2018). Furthermore, expression of genes associated with early and late osteogenic differentiation have been extensively studied (Hanna et al., 2018, Kulterer et al., 2007, Rutkovskiy et al., 2016, Stein et al., 2004). Therefore, to evaluate further the effect of mild perturbations to proteostasis, I used conventional methods and gene expression analysis after osteogenic differentiation.

3.3.1 VCP/p97 and proteasome inhibition do not alter matrix mineralisation as determined by standard methods

To study the effects of VCP/p97 and proteasome inhibition on matrix mineralisation, I first used conventional Alizarin S staining (Figure 20a, b and Table 4) and colorimetric calcium content quantitation (Figure 20c and Table 5), and although subtle qualitative changes in staining patterns were sometimes evident, I found that neither DBeQ nor bortezomib treatment resulted in significant gross changes in the formation of mineralised nodules.

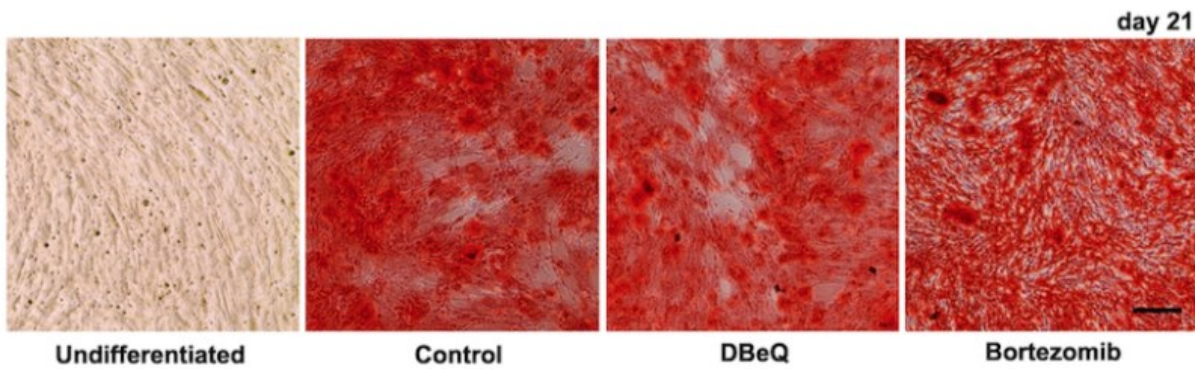
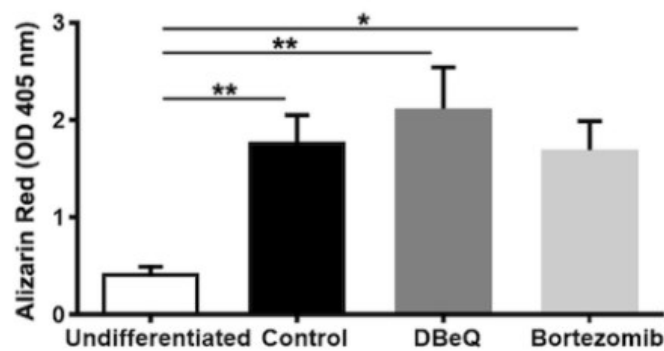
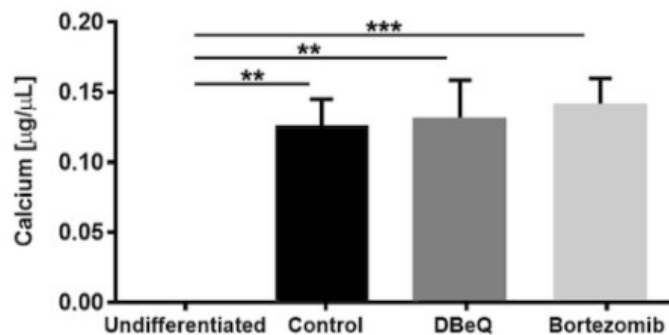
a**b****c**

Figure 20. VCP/p97 and proteasome inhibition do not alter matrix mineralisation as determined by standard methods. (a) Representative micrographs showing Alizarin Red S staining (Scale bar = 200 μm), (b) Alizarin Red S quantification and (c) colorimetric calcium content quantification of hMSC cultures differentiated for 21 days with DBeQ or bortezomib treatment on days 7, 10, 14 and 17. Plots show mean + SEM ($n=3$) and Kruskal-Wallis non-parametric test followed by Dunn's Multiple Comparison test to detect statistical significance, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Reproduced from (Loaiza et al., 2018) under the terms of the Creative Commons Attribution Non-Commercial 4.0 International Public License

Table 4. Statistical analyses of Alizarin Red S staining quantification in undifferentiated hMSC cultured for 21 days in medium without osteogenic agents and hMSC undergoing differentiation with DBeQ or bortezomib treatment on days 7, 10, 14 and 17 or vehicle control.

Alizarin Red S staining quantification	
Undifferentiated vs. control	$p = 0.003$
Undifferentiated vs. DBeQ	$p = 0.001$
Undifferentiated vs. bortezomib	$p = 0.013$
control vs. DBeQ	$p > 0.999$
control vs. bortezomib	$p > 0.999$
DBeQ vs. bortezomib	$p > 0.999$

Statistical analyses were carried out using a non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test. $n = 9$, from 3 different donors.

Table 5. Statistical analyses of colorimetric calcium content quantitation in undifferentiated hMSC cultured for 21 days in medium without osteogenic agents and hMSC undergoing differentiation with DBeQ or bortezomib treatment on days 7, 10, 14 and 17 or vehicle control.

Colorimetric calcium content quantitation	
Undifferentiated vs. control	$p = 0.002$
Undifferentiated vs. DBeQ	$p = 0.003$
Undifferentiated vs. bortezomib	$p = 0.001$
control vs. DBeQ	$p > 0.999$
control vs. bortezomib	$p > 0.999$
DBeQ vs. bortezomib	$p > 0.999$

Statistical analyses were carried out using a non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test. $n = 9$, from 3 different donors.

3.3.2 Mild proteostasis perturbations do not grossly affect the osteogenic differentiation of hMSC

I set out to quantify the expression of runt-related transcription factor 2 (*RUNX2*), alkaline phosphatase (*ALPL*), integrin binding sialoprotein (*IBSP*; also known as bone sialoprotein) and *SPARC* (secreted protein acidic and cysteine rich; also known as osteonectin), key osteogenic genes expressed during different stages of osteogenic differentiation. I chose to evaluate the expression of *RUNX2*, a transcription factor expressed throughout the differentiation process that regulates the expression of several other osteoblast-associated genes, including collagen type 1, osteocalcin and bone sialoprotein (Blair et al., 2017, Komori, 2010, Komori, 2019). As previously mentioned, alkaline phosphatase is a biochemical osteoblast marker increased with the duration of osteogenic differentiation. Upon differentiation, osteoblasts synthesise large quantities of extracellular matrix proteins, including osteonectin, a non-collagenous protein involved in mineralisation (Blair et al., 2017, Komori, 2019). Bone sialoprotein is also a component of mineralised tissue and therefore a marker of late differentiation.

The data obtained by gene expression analysis showed the expected expression profile of the osteoblast phenotype (Figure 21). *RUNX2* was upregulated during the whole differentiation period. *ALPL* showed the expression level progression with its expression starting to increase at day 7 of the differentiation phase. *IBSP* and *SPARC* expression achieved maximum level at day 21 of differentiation as they are involved in the matrix mineralisation phase.

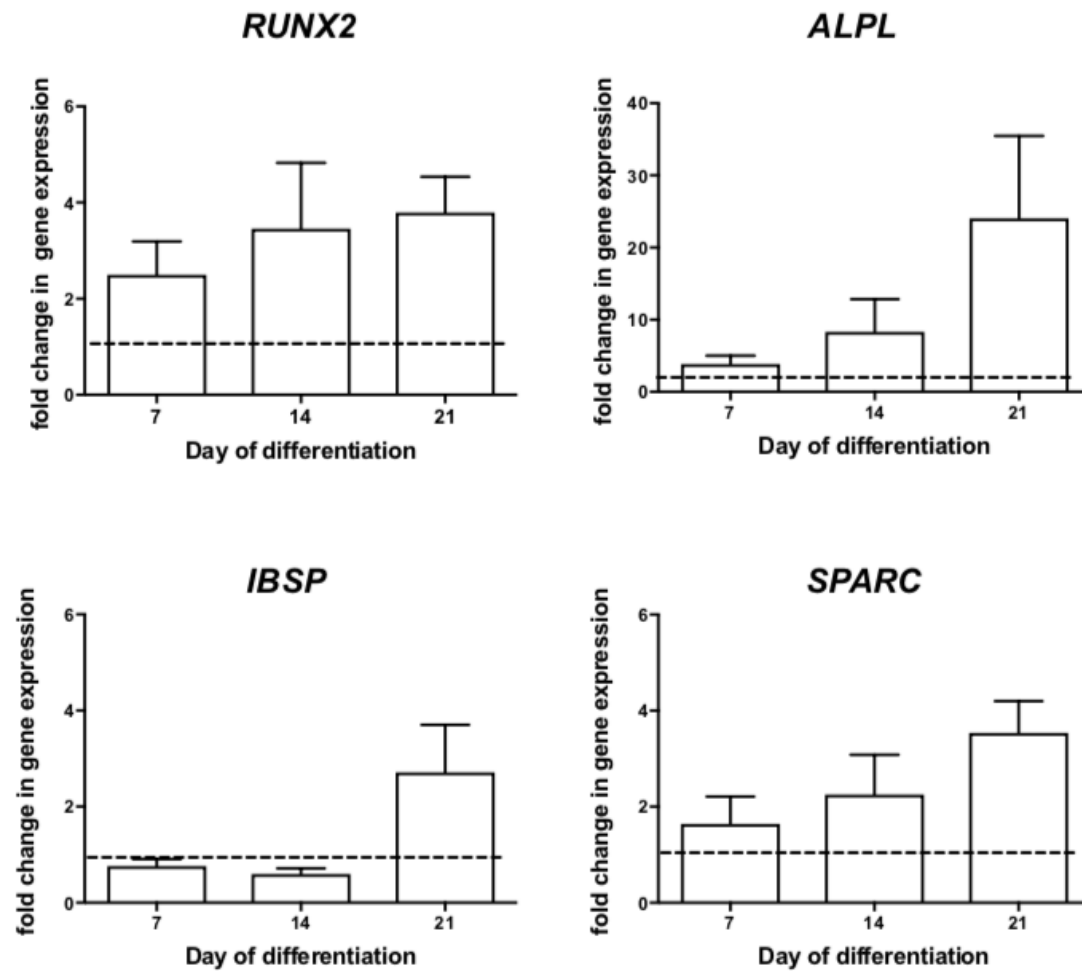


Figure 21. Osteogenic gene expression during differentiation of hMSC. Relative mRNA expression levels for osteogenic markers in differentiating hMSC on days 7, 14 and 21 compared to the undifferentiated control which is set to 1 (dotted horizontal line). Plots show mean + SEM ($n=3$).

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To study the effects of VCP/p97 and proteasome inhibition on matrix mineralisation further, I quantified the expression of genes associated with osteogenic differentiation and found that mRNA levels of *RUNX2*, *ALPL*, *IBSP* and *SPARC* were not significantly affected by treatment with DBeQ or bortezomib (Figure 22 and Table 6). Taken together, the data suggests that mild VCP/p97 or proteasome inhibition did not grossly affect the formation of bone-like mineralised nodules nor the expression of genes known to be key drivers of osteogenic differentiation *in vitro*.

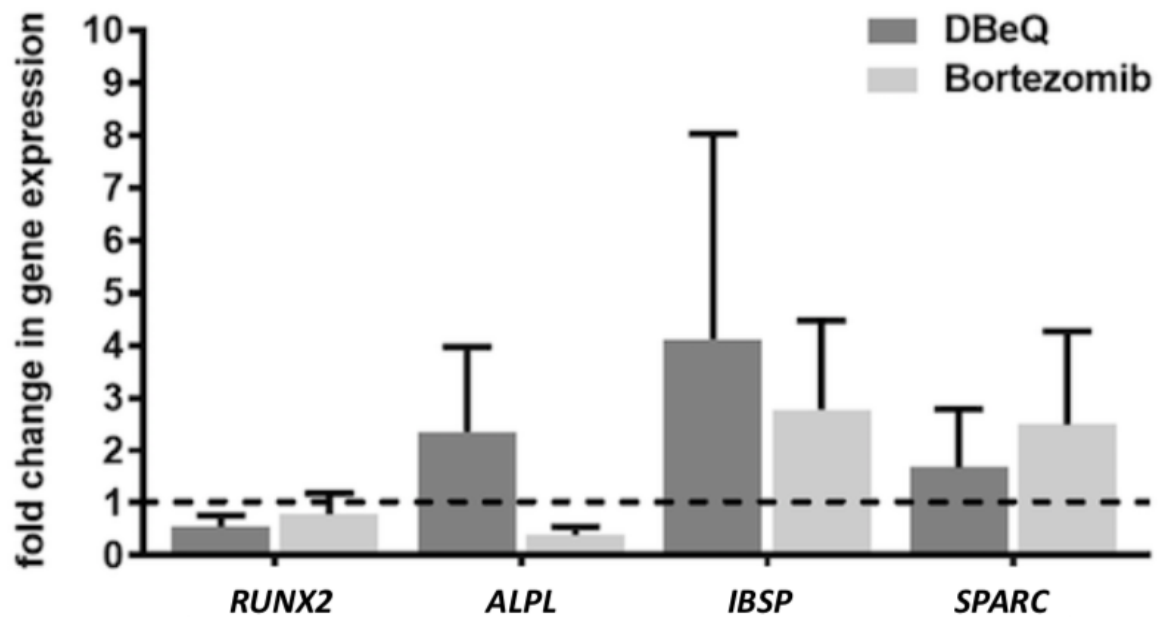


Figure 22. Mild proteostasis perturbations do not grossly affect osteogenic differentiation of hMSC. Relative mRNA expression levels for markers of osteogenic differentiation of hMSC cultures differentiated for 21 days with DBeQ or bortezomib treatment on days 7, 10, 14 and 17, compared to undifferentiated controls, which are set to 1 (dotted horizontal line). Plots show mean + SEM ($n=3$) and Kruskal-Wallis non-parametric test followed by Dunn's Multiple Comparison test to detect statistical significance, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

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Table 6. Statistical analyses of fold changes in osteogenic gene mRNA levels. hMSC undergoing osteogenic differentiation were treated with DBeQ, bortezomib or vehicle (control) on days 7, 10, 14 and 17 and mRNA was extracted on day 21.

	control vs. DBeQ	control vs. bortezomib	DBeQ vs. bortezomib
<i>RUNX2</i>	$p = 0.286$	$p = 0.866$	$p > 0.999$
<i>ALPL</i>	$p > 0.999$	$p = 0.388$	$p = 0.286$
<i>BSP</i>	$p > 0.999$	$p > 0.999$	$p > 0.999$
<i>ON</i>	$p > 0.999$	$p > 0.999$	$p > 0.999$

?

Statistical analyses were carried out using a non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test $n=3$, from 3 different donors.

3.4 Proteasome, but not VCP/p97 inhibition, subtly promotes mineralised matrix formation

Gross observations of osteogenesis are qualitative and can fail to take into account more subtle characteristics of bone formation and structure (Gentleman et al., 2009). To generate quantitative measures of the effects of proteostasis imbalances on tissue function, a collaboration with Dr. Eileen Gentleman from King's College Hospital, Martin A.B. Hedegaard from the University of Southern Denmark in Odense, Laurent Bozec and Sergio Bertazzo from University College London was established. All the experimental work was carried out by myself or with the collaborators and their teams.

3.4.1 VCP/p97 inhibition alters mineralised nodule composition as determined by Raman spectroscopy

We next applied material characterisation techniques to analyse the impact of VCP/p97 and proteasome inhibition on differentiating hMSC's ability to form a bone-like material *in vitro*. Raman spectroscopy detects vibrational bonds, capturing the 'biochemical signature' of matter. Raman spectroscopy has been used to characterise both the composition of native ECM (Tarnowski et al., 2003) and that secreted by cultured cells (Evans et al., 2012, Gentleman et al., 2009, Volponi et al., 2015). Raman spectra collected from mineralised nodules formed under all conditions revealed a sharp peak at $\sim 960\text{ cm}^{-1}$ indicative of mineral phosphate ion vibrations ($PO_4^{3-}\nu_1$) and other spectral features characteristic of native bone (Figure 23a) (Carden and Morris, 2000). This observation confirmed that all groups mostly formed a bone-like material as previously described (Evans et al., 2012, Gentleman et al., 2009, Volponi et al.,

2015). We then examined the integrated area of the PO_4^{3-} ν_1 peak, a relative measure of the amount of bone-like apatite (Tarnowski et al., 2003). Using standard univariate peak analysis, we observed an increase (although not statistically significant, $p = 0.085$) in the ν_1 PO_4^{3-} peak area in bortezomib-treated cultures compared to controls (Figure 24c and Table 7). VCP/p97 inhibition, on the other hand, did not alter the intensity of the PO_4^{3-} ν_1 peak. This suggests that whilst VCP/p97 inhibition did not affect the amount of mineral produced by hMSC, proteasome inhibition subtly enhanced it, a finding compatible with reports of the anabolic effects of proteasome inhibitors on bone (Gioia et al., 2012, Mukherjee et al., 2008, Qiang et al., 2009, Uyama et al., 2012b), particularly in multiple myeloma (Deleu et al., 2009, Giuliani et al., 2007, Lund et al., 2010).

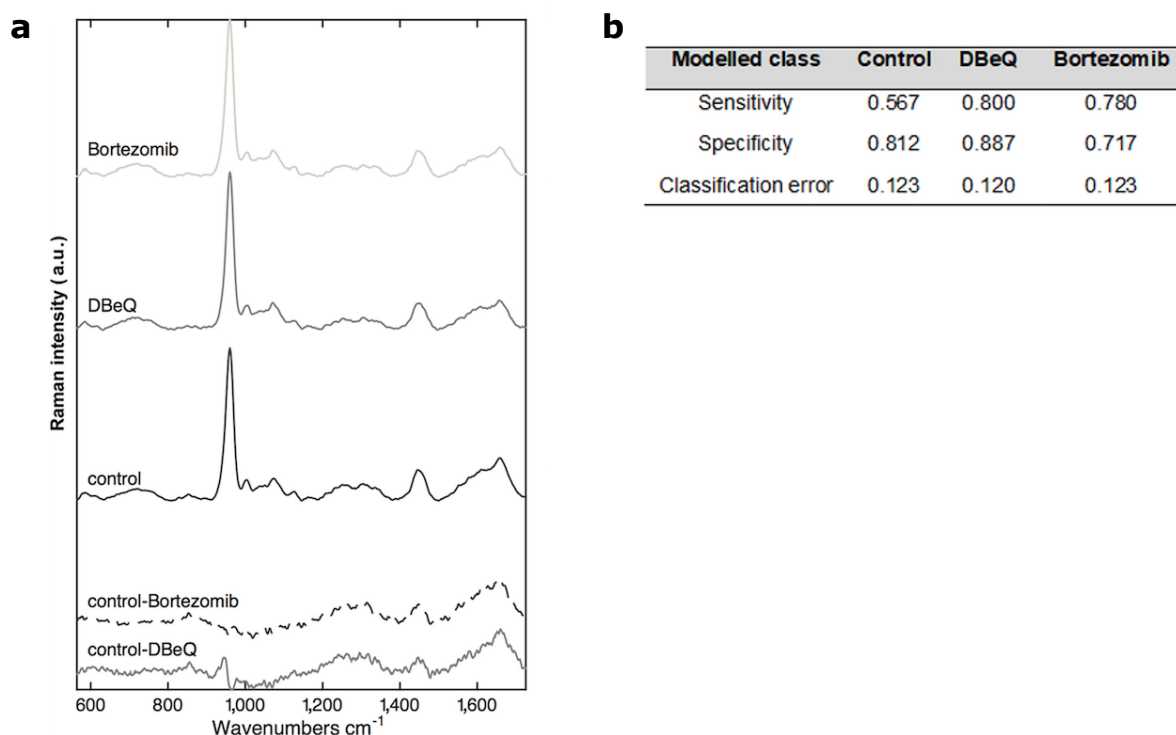


Figure 23. VCP/p97 inhibition alters mineralised nodule composition as determined by Raman spectroscopy. (a) Mean Raman spectra collected from mineralised nodules formed in control, DBeQ- and bortezomib-treated hMSC cultures. Difference spectra of DBeQ- and bortezomib-treated cultures compared to controls are shown in the bottom of the panel. Spectra are off-set in the y-axis for clarity. **(b)** Table showing the fraction of spectra in DBeQ- and bortezomib-treated groups and the control that could be classified by sensitivity, specificity and classification error using a cross-validated 6-component Partial Least Squares-Discriminant Analysis model.

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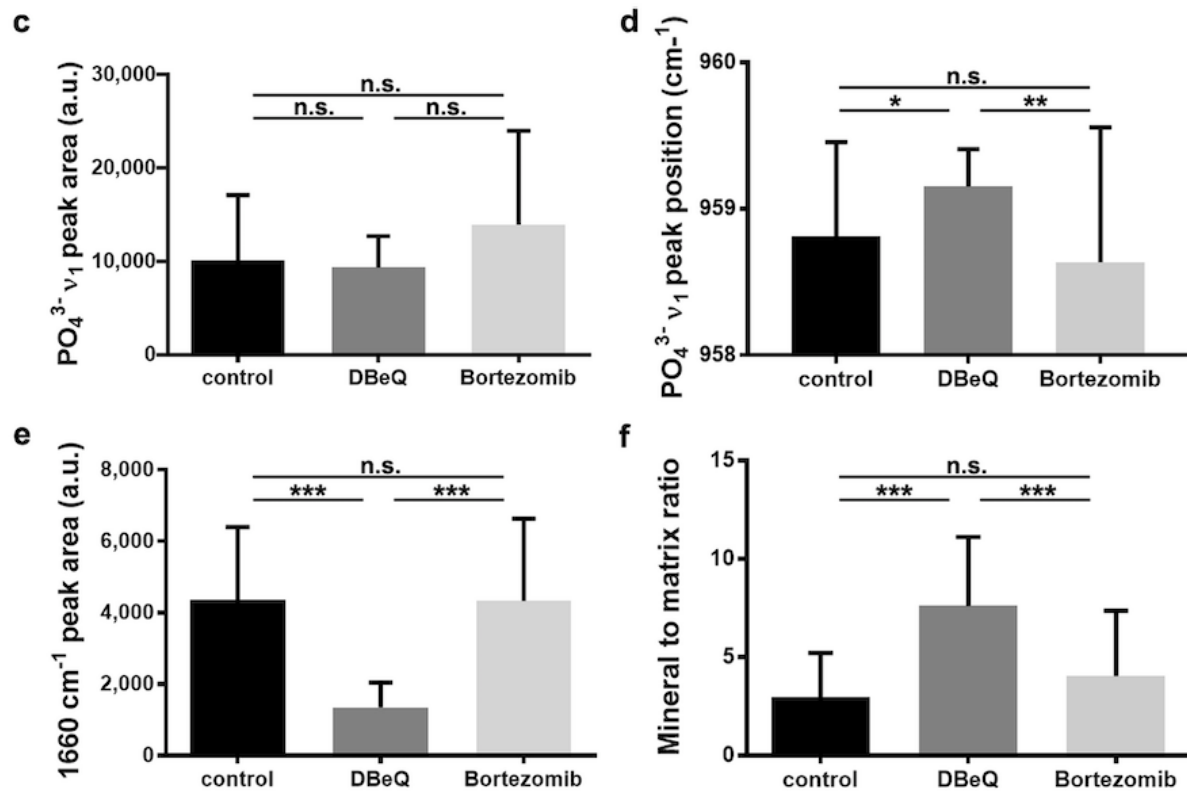


Figure 24. VCP/p97 inhibition alters mineralised nodule composition as determined by Raman spectroscopy. (c) Univariate analyses of the mean peak area in control, DBeQ- and bortezomib-treated cultures, (d) mean $\text{PO}_4^{3-} \nu_1$ peak position, and (e) 1660 cm^{-1} peak area. (f) Mean mineral to matrix ratio in control, DBeQ- and bortezomib-treated cultures. In c-f, data are means +SD and Kruskal-Wallis non-parametric test followed by Dunn's Multiple Comparison test was used to detect statistical significance. * $p < 0.05$, ** $p < 0.01$ and *** $p=0.001$. For detailed n and p values see Table 7.

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Table 7. Statistical analyses of univariate analyses of the mean PO_4^{3-} ν_1 peak area at $\sim 960\text{ cm}^{-1}$, mean PO_4^{3-} ν_1 peak position, 1660 cm^{-1} peak area and mean mineral to matrix ratio in control, DBeQ- and bortezomib-treated cultures.

PO_4^{3-} ν_1 peak area		
control ($n = 70$) vs. DBeQ ($n = 62$) K-W, D: $p > 0.999$	control ($n = 70$) vs. bortezomib ($n = 85$) K-W, D: $p = 0.085$	DBeQ ($n = 62$) vs. bortezomib ($n = 85$) K-W, D: $p = 0.150$
PO_4^{3-} ν_1 peak position		
control ($n = 80$) vs. DBeQ ($n = 64$) K-W, D: $p = 0.013$	control ($n = 80$) vs. bortezomib ($n = 86$) K-W, D: $p > 0.999$	DBeQ ($n = 64$) vs. bortezomib ($n = 86$) K-W, D: $p = 0.005$
1660 cm^{-1} peak area		
control ($n = 76$) vs. DBeQ ($n = 64$) K-W, D: $p < 0.001$	control ($n = 76$) vs. bortezomib ($n = 79$) K-W, D: $p > 0.999$	DBeQ ($n = 64$) vs. bortezomib ($n = 79$) K-W, D: $p < 0.001$
Mineral to matrix ratio		
control ($n = 78$) vs. DBeQ ($n = 59$) K-W, D: $p < 0.001$	control ($n = 78$) vs. bortezomib ($n = 80$) K-W, D: $p = 0.158$	DBeQ ($n = 59$) vs. bortezomib ($n = 80$) K-W, D: $p < 0.001$

Statistical analyses were carried out using a non-parametric Kruskal-Wallis (K-W) test followed by Dunn's (D) multiple comparison test. All n values were obtained after excluding outliers using a ROUT test.

3.4.2 VCP/p97, but not proteasome inhibition, impacts spectroscopic measures of mineral crystallinity and decreases proteinaceous matrix deposition

Raman spectral analyses not only provide information about the relative quantity of a substance, but also its structure. Therefore, we next analysed PO_4^{3-} ν_1 peak position as an indicator of mineral crystallinity. A downward shift is associated with a more amorphous, disorganised apatite whereas an upward shift suggests that the mineral is more crystalline (Tarnowski et al., 2003). Mean ν_1 PO_4^{3-} peak position for bortezomib-treated hMSC was similar to that of controls. However, in the DBeQ-treated group, it was significantly higher (Figure 24d and Table 7). A shift in PO_4^{3-} ν_1 peak position was also evident in difference spectra generated by comparing mean DBeQ- and bortezomib-treated conditions to controls (Figure 23a, bottom-most spectra). The 'control-DBeQ' spectrum produced a shift at $\sim 960\text{ cm}^{-1}$, whilst no shift was evident in the 'control-bortezomib' difference spectrum.

As VCP/p97 inhibition appeared to impact nodules' mineral crystallinity, we next asked if the proteinaceous component had also been affected. The Raman peak centred $\sim 1660\text{ cm}^{-1}$ has been attributed to Amide I and is an indication of protein content (Tarnowski et al., 2003). Bortezomib treatment did not affect the integrated area of the Amide I peak; however, it was significantly lower in DBeQ-treated nodules compared to controls (Figure 24e and Table 7), suggesting that VCP/p97 inhibition decreased the amount of Amide I-containing protein secreted by hMSC. This was reflected in mineral to matrix ratio, a measure of the relative amount of apatite to protein nodules. Whilst bortezomib-treated nodules were no different, DBeQ treatment produced

nodules with significantly higher mineral to matrix ratios compared to controls (Figure 24f and Table 7).

next aimed to confirm the Raman spectroscopic distinctiveness of nodules formed when hMSC subjected to VCP/p97 as opposed to proteasome inhibition in multi-variate analysis techniques. Using a 6-component Partial Least Squares-Discriminant Analysis model, we found that whilst all groups showed similar classification error, we were able to classify a larger fraction of DBeQ-treated spectra with better sensitivity and specificity than either control or bortezomib-treated groups (Figure 23b). This suggests that whilst control and bortezomib-treated mineralised nodules were similar spectroscopically, DBeQ treatment produced spectra that were more unique and so amenable to classification in the model. Taken together, these observations suggest not only that VCP/p97 inhibition affected the composition of the cell-derived mineralised material created by differentiating hMSC, but also highlights that these effects were quantifiable using a relatively simple interdisciplinary technique.

3.5 Proteasome and VCP/p97 inhibition have differing effects on mineralised nodules' stiffness and adhesion interactions

3.5.1 VCP/p97, but not the proteasome inhibition, decreases mineralised nodule stiffness

As VCP/p97 inhibition affected the biochemical composition of mineralised nodules, we next aimed to expand our *in vitro* model and determine its effect on nodules' nano-scale mechanical properties by measuring their stiffness. AFM is known for its high-resolution imaging capabilities, however, it is also a powerful tool for carrying out force-indentation measurements (Figure 25a and b), producing quantitative insight into the mechanical properties of a material at the nano-scale (Choi et al., 2019). We found that Young's modulus (E) was significantly lower in nodules treated with DBeQ compared to controls or those treated with bortezomib (Figure 25c and Table 8). As indentation was carried out with a probe whose tip radius was 8 nm (manufacturer's specification), this observation suggests that VCP/p97 inhibition significantly reduced stiffness of the cell-derived material created by differentiating hMSC at the scale of the nano-composite structure of native bone.

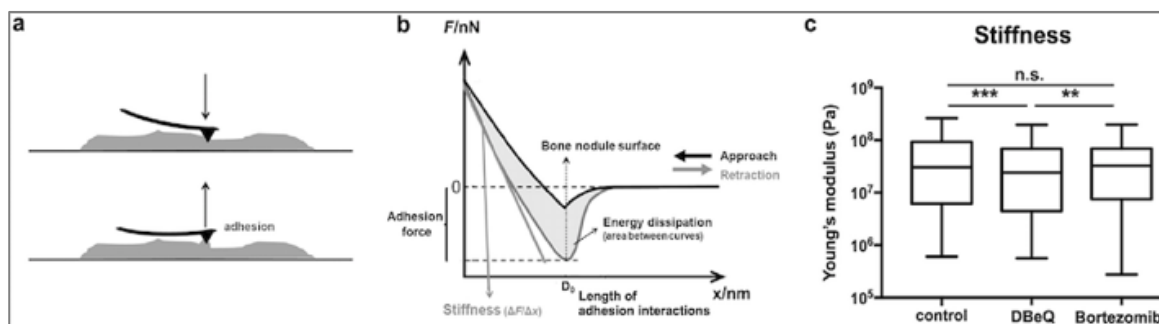


Figure 25. Proteasome and VCP/p97 inhibition have differing effects on mineralised nodules' stiffness and adhesion interactions. (a) Schematic showing how mineralised nodules were probed by AFM in force-indentation mode using a cantilever with a pyramidal tip. **(b)** Typical force-indentation curve generated from indenting a mineralised nodule. Schematic shows how nodule stiffness and adhesion interactions were calculated from the retraction curve. **(c)** Measurements of Young's Modulus (Pa) of mineralised nodules. Plots show medians, 1st and 3rd quartiles and highest and lowest values. A non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison was used to determine statistical significance. Significant differences in the distributions of adhesion values were evaluated using a Mantel-Haenszel linear-by-linear association Chi-squared (χ^2) test for trend. Power was evaluated by determining Goodman and Kruskal's gamma (γ). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. For detailed n and p values see Table 8.

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3.5.2 Proteasome inhibition reduces the strength of adhesion interactions, but VCP/p97 inhibition enhances the total energy dissipated

In addition to probing their stiffness, AFM-based indentation measurements also reveal information regarding the cell-derived materials' proteinaceous content. This is because upon indentation, the AFM probe forms non-specific adhesion interactions with the surface of the nodule (likely with proteins). During unloading of the indentation measurement, the deflection of the cantilever can reveal strength of the adhesion interactions between the probe and the sample, as well as measure the distance over which those adhesion interactions take place. We first found that adhesion force (Figure 26 and Table 8) was not different in DBeQ-treated samples compared to controls; however in bortezomib-treated samples, it was significantly lower. This suggests that bortezomib-treated nodules interacted with the AFM probe less strongly, likely because the protein composition was different and/or was tightly bound to the more highly mineralised nodules and thus less available to interact with the AFM probe.

We next studied the length of the adhesion interactions, a measure of the distance over which adhesion interactions take place between the sample and the AFM probe during the unloading phase (Figure 27 and Table 8). DBeQ treatment produced interactions at significantly larger adhesion interactions lengths than those observed in either control or bortezomib-treated nodules. Analyses of the distribution showed that in DBeQ-treated nodules, interactions frequently took place as the probe retracted several microns from the contact point (2-4 μm), as confirmed by statistical significant analyses for trend (Figure

27 and Table 8). This contrasts with measurements on control and bortezomib-treated cultures, where most interactions took place within a few hundred nanometers of the contact point. This was also evident in measurements of total adhesion energy, which accounts for both adhesion strength and distance over which it acts (Figure 28 and Table 8). All groups showed interactions with the probe at low values for total energy (first peak in histogram at <20 fJ), likely reflecting interactions between the AFM probe and protein that was strongly bound to mineral. However, median adhesion energy was significantly higher in DBeQ-treated groups and was concomitant with many individual measurements at high values of energy dissipation. These observations suggest that VCP/p97 inhibition produced nodules that were highly compliant mechanically and whose protein may have been less tightly bound to mineral (or protein had different charge/adhesive properties), and thus could be extended away from the nodules and dissipate energy via interactions with the AFM probe. On the other hand, protein in control and bortezomib-treated nodules may have been more strongly bound to mineral, as in native bone. Moreover, these observations demonstrate that AFM, a widely-available technique, can identify quantifiable changes in tissue properties in response to mild VCP/p97 impairment.

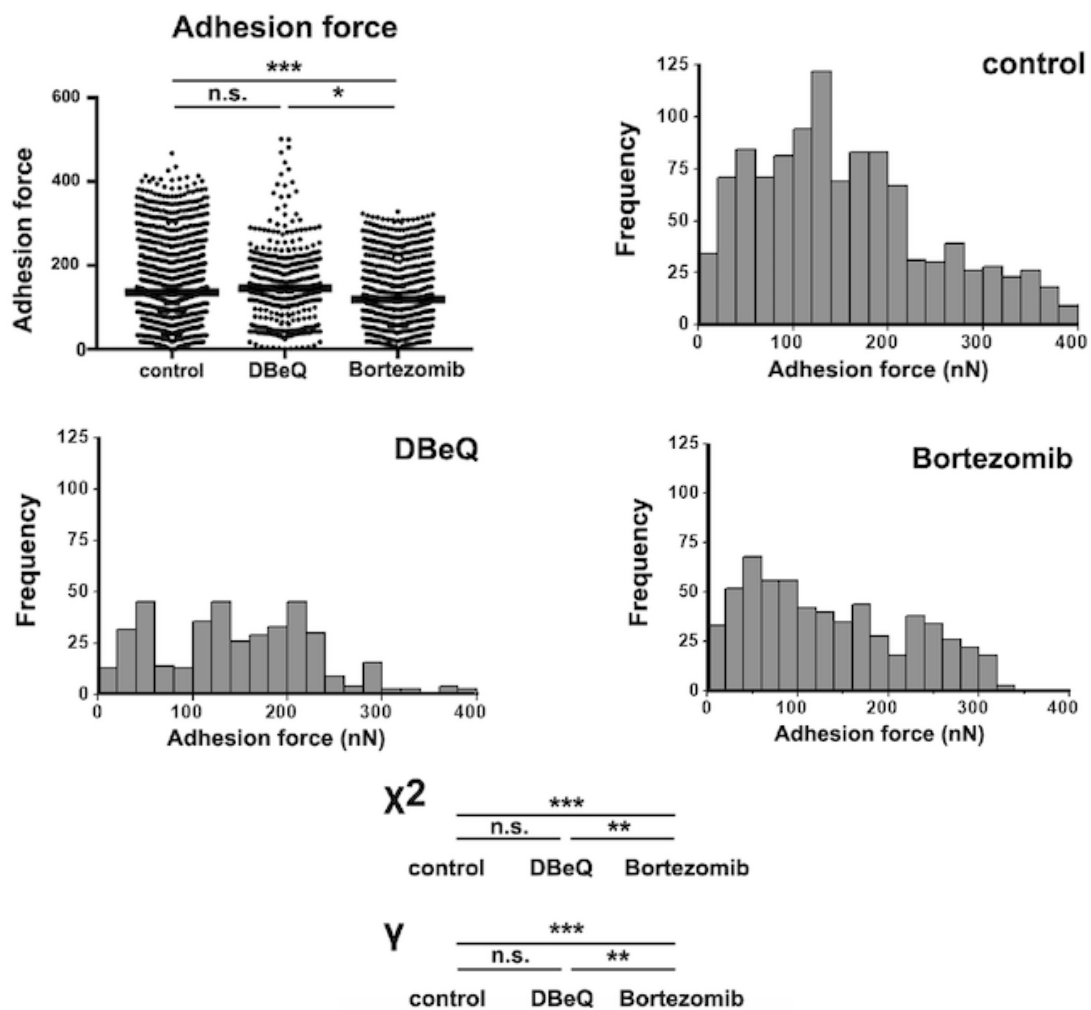


Figure 26. Proteasome and VCP/p97 inhibition have differing effects on mineralized nodules' adhesion force. Measurements of adhesion force from force-indentation curves on control, DBeQ- and bortezomib-treated cultures. Plots show medians. Histograms with their associated statistical analyses show how the distributions of values differed between the groups. A non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison was used to determine statistical significance. Significant differences in the distributions of adhesion values were evaluated using a Mantel-Haenszel linear-by-linear association Chi-squared (χ^2) test for trend. Power was evaluated by determining Goodman and Kruskal's gamma (γ). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. For detailed n and p values see Table 8.

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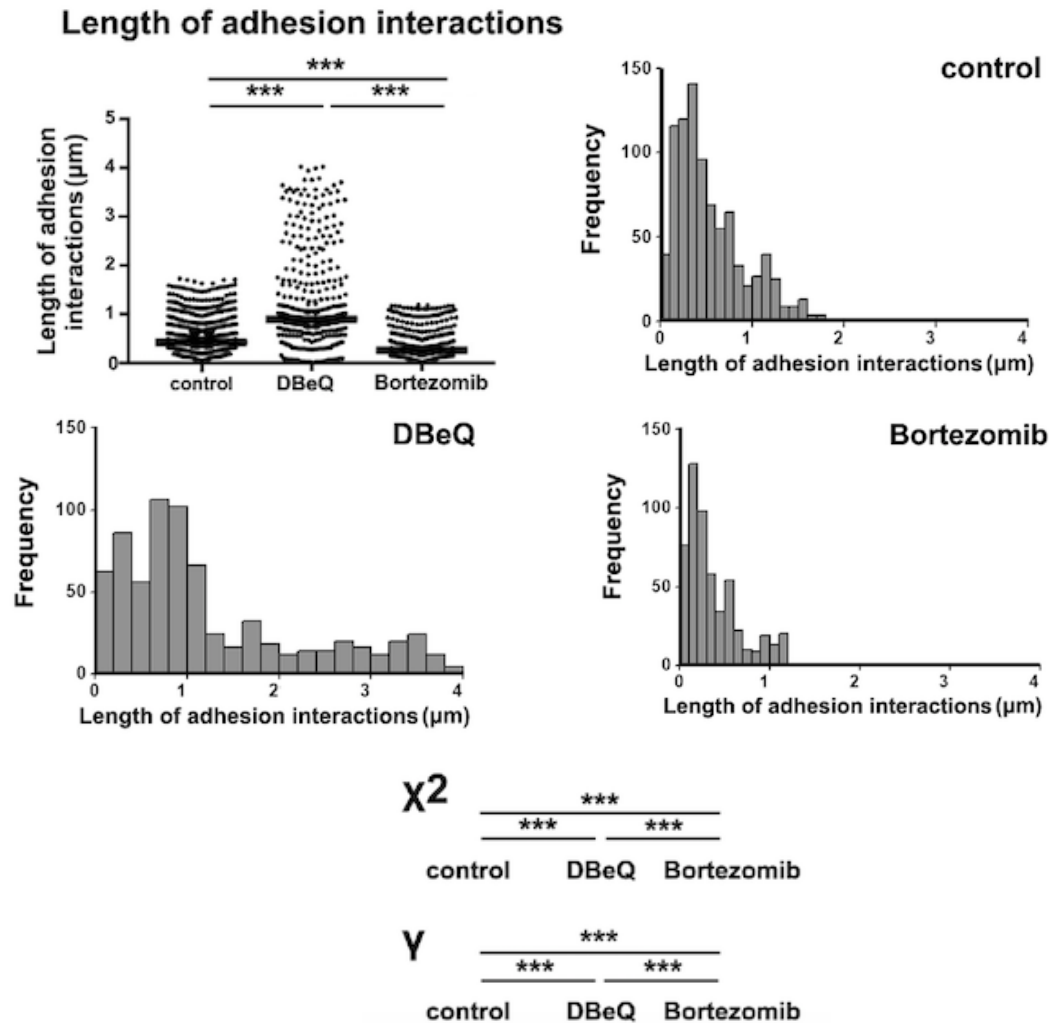


Figure 27. Proteasome and VCP/p97 inhibition have differing effects on mineralized nodules' adhesion interactions. Measurements of length of adhesion interactions from force-indentation curves on control, DBeQ- and bortezomib-treated cultures. Plots show medians. Histograms with their associated statistical analyses show how the distributions of values differed between the groups. A non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison was used to determine statistical significance. Significant differences in the distributions of adhesion values were evaluated using a Mantel-Haenszel linear-by-linear association Chi-squared (χ^2) test for trend. Power was evaluated by determining Goodman and Kruskal's gamma (γ). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. For detailed n and p values see Table 8.

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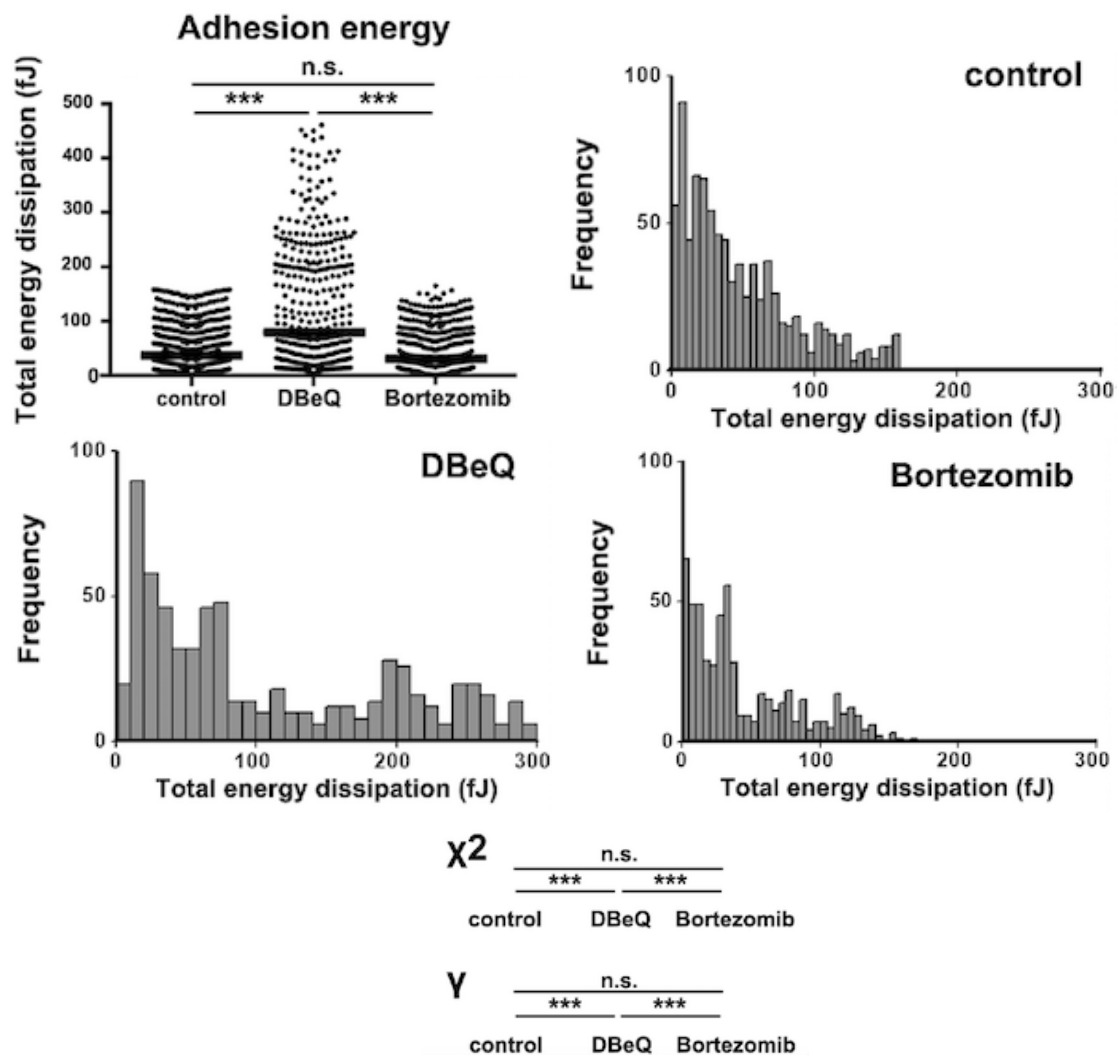


Figure 28. Proteasome and VCP/p97 inhibition have differing effects on mineralised nodules' adhesion energy. Measurements of adhesion energy generated from force-indentation curves on control, DBeQ- and bortezomib-treated cultures. Plots show medians. Histograms with their associated statistical analyses show how the distributions of values differed between the groups. A non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison was used to determine statistical significance. Significant differences in the distributions of adhesion values were evaluated using a Mantel-Haenszel linear-by-linear association Chi-squared (χ^2) test for trend. Power was evaluated by determining Goodman and Kruskal's gamma (γ). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. For detailed n and p values see Table 8.

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Table 8. Statistical analyses of distributions of stiffness (*E*, Young's modulus), adhesion force, pulling length, and energy dissipation in bone nodules.

Young's modulus		
Control (<i>n</i> = 2090) vs. DBEq (<i>n</i> = 1831)	Control (<i>n</i> = 2090) vs. bortezomib (<i>n</i> = 1516)	DBEq (<i>n</i> = 1831) vs. bortezomib (<i>n</i> = 1516)
K-W, D: <i>p</i> < 0.001	K-W, D: <i>p</i> = 0.068	K-W, D: <i>p</i> = 0.003
$\chi^2 = 79.699$, <i>p</i> < 0.001 $\gamma = -0.107$, <i>p</i> < 0.001	$\chi^2 = 49.144$, <i>p</i> < 0.001 $\gamma = -0.041$, <i>p</i> = 0.059	$\chi^2 = 3.248$, <i>p</i> = 0.071 $\gamma = 0.069$, <i>p</i> = 0.003
SR (Pa): 6.1×10^7 — 7×10^7 , 8.1×10^7 — 9×10^7 , 1.11×10^8 — 1.2×10^8 , 1.81×10^8 — 2.5×10^8	SR (Pa): 1.1×10^7 — 3×10^7 , 6.1×10^7 — 7×10^7 , 8.1×10^7 — 9×10^7 , 1.81×10^8 — 1.9×10^8	SR (Pa): $< 1 \times 10^7$ — 3×10^7 , 5.1×10^7 — 6×10^7
Adhesion force		
Control (<i>n</i> = 1102) vs. DBEq (<i>n</i> = 411)	Control (<i>n</i> = 1102) vs. bortezomib (<i>n</i> = 613)	DBEq (<i>n</i> = 411) vs. bortezomib (<i>n</i> = 613)
K-W, D: <i>p</i> > 0.999	K-W, D: <i>p</i> < 0.001	K-W, D: <i>p</i> = 0.012
$\chi^2 = 0.580$, <i>p</i> = 0.446 $\gamma = -0.007$, <i>p</i> = 0.842	$\chi^2 = 20.690$, <i>p</i> < 0.001 $\gamma = -0.129$, <i>p</i> < 0.001	$\chi^2 = 9.121$, <i>p</i> = 0.003 $\gamma = -0.104$, <i>p</i> = 0.007
SR (N): none	SR (N): $< 2 \times 10^{-8}$, 1.2×10^{-7} — 1.4×10^{-7} , 1.8×10^{-7} — 2×10^{-7} , 2.2×10^{-7} — 2.6×10^{-7} , 3.2×10^{-7} — 3.8×10^{-7}	SR (N): 6×10^{-8} — 1×10^{-7} , 1.2×10^{-7} — 1.4×10^{-7} , 1.8×10^{-7} — 2.2×10^{-7} , 2.4×10^{-7} — 2.6×10^{-7}
Adhesion position		
Control (<i>n</i> = 887) vs. DBEq (<i>n</i> = 360)	Control (<i>n</i> = 887) vs. bortezomib (<i>n</i> = 541)	DBEq (<i>n</i> = 360) vs. bortezomib (<i>n</i> = 541)
K-W, D: <i>p</i> < 0.001	K-W, D: <i>p</i> < 0.001	K-W, D: <i>p</i> < 0.001
$\chi^2 = 254.559$, <i>p</i> < 0.001 $\gamma = 0.509$, <i>p</i> < 0.001	$\chi^2 = 78.046$, <i>p</i> < 0.001 $\gamma = -0.352$, <i>p</i> < 0.001	$\chi^2 = 257.383$, <i>p</i> < 0.001 $\gamma = -0.706$, <i>p</i> < 0.001
SR (m): $< 2 \times 10^{-7}$ — 6×10^{-7} , 1×10^{-6} — 1.2×10^{-6} , 1.8×10^{-6} — 3.6×10^{-6}	SR (m): $< 2 \times 10^{-7}$, 6×10^{-7} — 8×10^{-7} , 1.2×10^{-6} — 1.6×10^{-6}	SR (m): $< 2 \times 10^{-7}$ — 1×10^{-6} , 1.2×10^{-6} — 3.6×10^{-6}
Adhesion energy		
Control (<i>n</i> = 858) vs. DBEq (<i>n</i> = 369)	Control (<i>n</i> = 2090) vs. bortezomib (<i>n</i> = 558)	DBEq (<i>n</i> = 369) vs. bortezomib (<i>n</i> = 558)
K-W, D: <i>p</i> < 0.001	K-W, D: <i>p</i> = 0.223	K-W, D: <i>p</i> < 0.001
$\chi^2 = 279.778$, <i>p</i> < 0.001 $\gamma = 0.487$, <i>p</i> < 0.001	$\chi^2 = 1.692$, <i>p</i> = 0.193 $\gamma = -0.072$, <i>p</i> = 0.064	$\chi^2 = 213.847$, <i>p</i> < 0.001 $\gamma = -0.525$, <i>p</i> < 0.001
SR (J): $< 2 \times 10^{-14}$ — 6×10^{-14} , 1.6×10^{-13} — 3×10^{-13}	SR (J): 4×10^{-14} — 6×10^{-14} , 1.4×10^{-13} — 1.6×10^{-13}	SR (J): $< 2 \times 10^{-14}$ — 4×10^{-14} , 1.8×10^{-13} — 3×10^{-13}

Statistical analyses were carried out using a non-parametric Kruskal-Wallis (K-W) test followed by Dunn's (D) multiple comparison test. A Mantel-Haenszel linear-by-linear association Chi-square test for trend (χ^2) (degrees of freedom = 1) was used to test for whether the distributions were significantly different from one another. A non-parametric Goodman and Kruskal's gamma (γ) test was then applied to measure of the strength of association that exists between any two comparisons. Low values for association indicate that two distributions are highly similar, while higher values are evidence of a stronger association and indicate that the distributions are different: none (0.00 – ± 0.01), weak association (± 0.01 – 0.09), moderate association (± 0.10 – 0.29), evident strong association (± 0.30 – 0.99). Standardized residuals (SR) highlight the most significant areas of the histograms that contributed to differences. All *n* values were obtained after excluding outliers with ROUT test.

3.6 VCP/p97, but not proteasome inhibition impacts the ultra- and micro-structure of mineralised nodules

As VCP/p97 inhibition affected the biochemical composition, as well as the mechanical and adhesive properties of the bone-like material formed by hMSC, we next aimed to visualise its effects on this matrix using a combination of transmission (TEM) and scanning electron microscopy (SEM). TEM micrographs of control and bortezomib-treated cultures had similar ultrastructures to those previously described for bone-like nodules (Bonewald et al., 2003, Gentleman et al., 2009), and were notable for aligned fibrous protein between cells (Figure 29). However, in the DBeQ-treated cultures, we often observed more disorganised matrix in the intercellular space, and aligned fibrous protein was less common. We also examined nodules by SEM using both backscatter and secondary electron modes. Combining the two to create density-dependent colour SEM (DDC-SEM) images allowed us to visualise dense mineralised and less dense proteinaceous areas (Bertazzo et al., 2013). hMSC cultured under basal conditions did not form mineralised nodules (Figure 30). Images of the material formed under control conditions and those treated with bortezomib contained dense, mineralised areas that appeared to closely associate with less dense matrix, with morphologies similar to those previously described (Querido et al., 2011). However, in DBeQ-treated cultures, although some dense material localised with matrix, we also observed highly dense areas with little to no associated less dense matrix. Moreover, the structure of the dense mineral DBeQ-treated cultures appeared different morphologically. Whilst dense mineralised areas of control cultures appeared smooth, mineral in

DBeQ-treated cultures appeared rougher, with needle-like crystals characteristic of non-physiological precipitation (Figure 31).

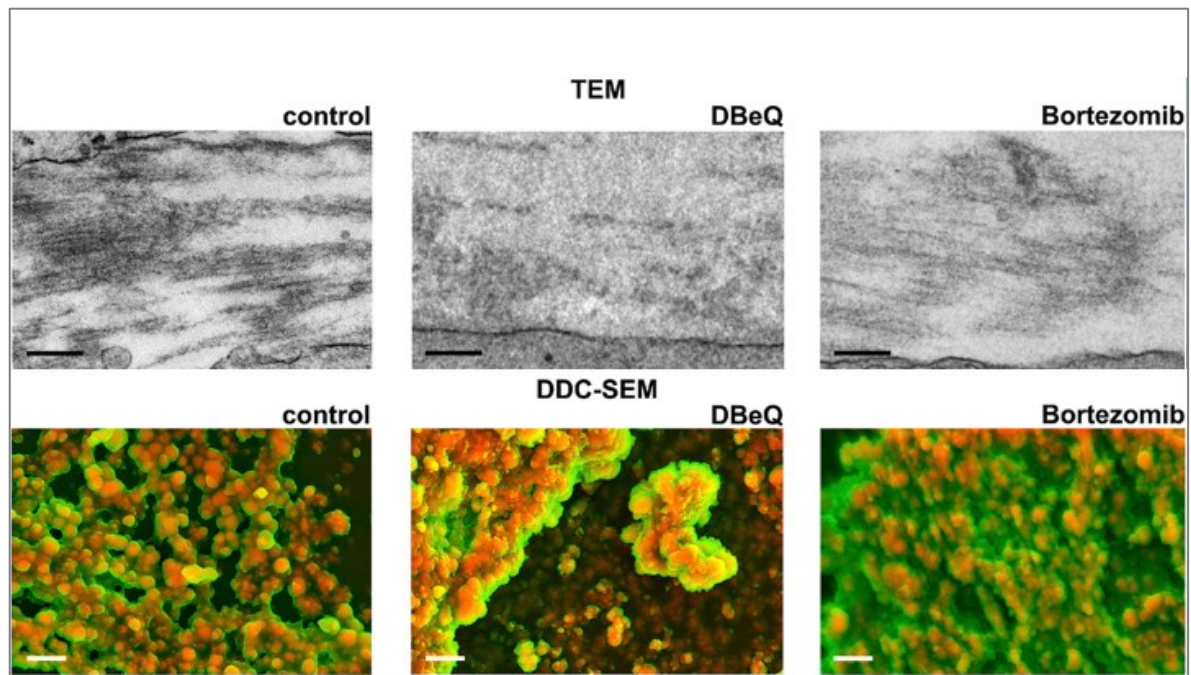


Figure 29. VCP/p97 inhibition impacts the ultra- and micro-structure of mineralised nodules. (a) Transmission electron microscopy (TEM) and density-dependent colour scanning electron microscopy (DDC-SEM) micrographs of mineralised nodules formed from hMSC under standard osteogenic conditions or treated with DBeQ or bortezomib. In TEM images, proteinaceous fibrils are evident in the intercellular space in control and bortezomib-treated cultures. In DBeQ-treated samples, the proteinaceous matrix between cells appeared amorphous and clear fibrils were often not evident. Scale bar = 250 nm. In DDC-SEM micrographs, images were coloured in post-processing by combining images from secondary and backscatter electron detectors to identify dense mineral (red) and less dense matrix (green). In control and bortezomib-treated groups, there appears to be an association between the organic matrix and the mineral. In DBeQ-treated groups, dense mineral was often detected without the associated presence of less-dense matrix. Images are representative from experiments carried out on cultures grown from 3 independent donors. Scale bar = 2 μ m.

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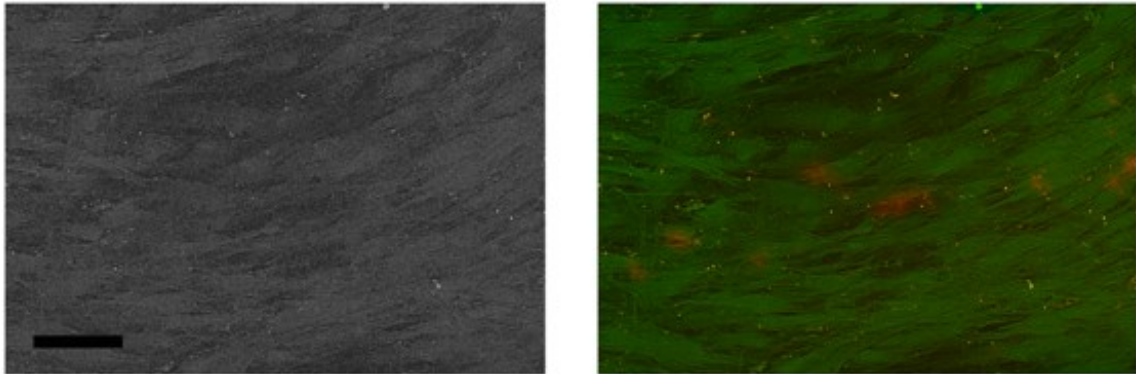


Figure 30. Backscatter and DDC-SEM micrographs of hMSC cultured for 21 days under basal conditions. hMSC not treated with osteogenic medium do not form mineralised nodules and DDC-SEM images appear almost entirely green. Scale bar = 100 μm .

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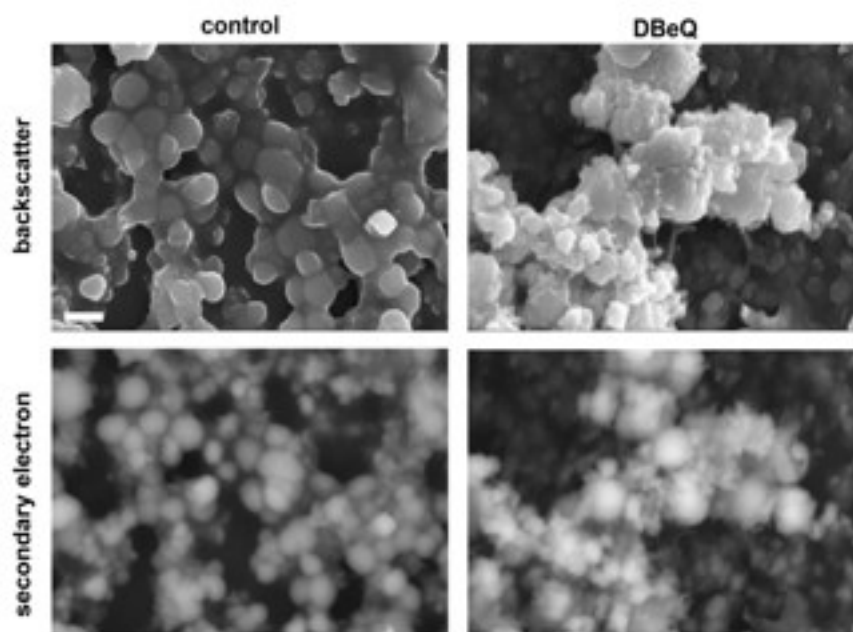


Figure 31. Backscatter and secondary electron images of mineralised nodules collected from control and DBeQ-treated cultures. Images show that whilst control cultures produce mineral that appears smooth, the mineral within DBeQ-treated cultures is often rougher, more plate- or needle-like and indicative of that produced by non-physiological precipitation reactions rather than a controlled biomineralisation process. Scale bar = 1 μ m.

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4 Discussion

I have used a combination of materials science-based characterisation approaches to show that subtle impairment of the intracellular protein degradation machinery, leading to mild intracellular stress, can modify tissue physical properties in a cell-derived *in vitro* model that mimics the formation of native bone tissue. Importantly, these techniques revealed an array of biophysical and biochemical changes that were missed by what one might call conventional approaches: both Alizarin Red S staining and colorimetric calcium quantification, two methods that are widely used in bone and mineralisation research, suggested that DBeQ- and bortezomib-treated as well as control cultures all produced a similar amount of mineralised material. However, Raman spectroscopy revealed that the composition of mineralised bone-like nodules and protein content in the extracellular matrix of the tissue formed *in vitro* was affected. By quantifying the $PO_4^{3-} \nu_1$ peak intensity of Raman spectra, I determined that bortezomib-treated samples produced numerically more apatite than other groups (although this was not statistically significant). Alizarin red S staining is a crude estimate of bone formation and whilst it detects the presence of calcium-containing compounds (Evans et al., 2010), bone mineral is more complex. Here, Raman spectral measurements were able to provide more specific information regarding bone-like tissue formation.

In my *in vitro* model, Raman spectroscopy identified quantitative effects of VCP/p97 inhibition, which contrasted with those of proteasome inhibition. Specifically, whilst DBeQ appeared to increase mineral crystallinity, bortezomib had no significant effect on spectroscopic measures of apatite structure. Mineral

crystallinity increases with age (Akkus et al., 2004) and has been associated with decreased bone ductility (Yerramshetty and Akkus, 2008) and increased fracture risk (Gourion-Arsiquaud et al., 2009), suggesting spectroscopic measurements may provide informative functional measurements of bone quality. VCP/p97 inhibition also significantly decreased nodule protein content, producing a higher mineral to matrix ratio when compared to controls. Bortezomib treatment subtly promoted mineral formation, and this was accompanied by appropriate formation of proteinaceous matrix, as mineral to matrix ratio was not different than that of controls.

As bone formation is a highly controlled process in which mineral templates on a proteinaceous matrix, this observation suggests that whilst bortezomib increased mineralisation (in line with previous observations) (Giuliani et al., 2007, Mukherjee et al., 2008, Uyama et al., 2012a), proteasome inhibition did not fundamentally interfere with this process. The findings also lend support to those who argue for a bone-anabolic effect of proteasome inhibitors in patients with multiple myeloma. However, it needs to be recognised that the *in vitro* system I used cannot recreate the complexity of *in vivo* interactions (including tumour cells and their microenvironment consisting of multiple different haematopoietic, immunological, osteogenic and angiogenic components). DBeQ treatment, on the other hand, induced the formation of mineral in the absence of appropriate proteinaceous matrix deposition, suggesting that the mineral may have been aberrant. As Raman spectral measurements are relatively straightforward and can be performed on almost any biological sample under any condition (wet/dry/fixed/unfixed/etc.) in the absence of labelling or staining, these observations highlight the technique's utility as a quantitative means to identify functional tissue effects of drugs in clinical development such as VCP/p97 inhibitors. Therefore, these observations may have implications for cancer

therapeutics that interfere with intracellular protein turnover (synthesis or degradation) and are of particular relevance to drugs that aim to target anti-neoplastic effect with treating osteolytic bone disease (such as, possibly, proteasome inhibitors or agents targeting primary bone cancers or bone metastases).

One of bone's primary roles is to resist applied load. Therefore, its mechanical properties, including stiffness, are central to its function. Indeed, pathological changes in mechanical properties are associated with diseases including osteogenesis imperfecta, osteopetrosis, osteoporosis and rickets (Li and Aspden, 1997). The AFM-based indentation measurements I carried out indicated that whilst bortezomib had no effect on the cell-derived material's stiffness, DBeQ significantly decreased their Young's modulus. Differences in stiffness are likely not attributable to cultures forming less mineral, as both histochemical staining and spectroscopic analyses showed that VCP/p97 inhibition did not reduce the amount of mineral compared to control cultures. Instead, my observations suggest that DBeQ altered either the composition or structure of mineralised nodules. In bone, the protein-mineral nano-composite provides toughness via pre-stress within its structure. This is mediated by cross-linked collagen fibres, which impart compressive stress on mineral crystals as they form, whilst the growing mineral crystals tense the collagen structure (Reznikov et al., 2016). My observations that DBeQ-treated nodules were less stiff suggests that these composite interactions may have been limited.

My AFM-based indentation measurements also produced highly informative observations with regards to adhesion interactions. Adhesion interactions with the AFM probe can be mediated by non-specific interactions, likely with protein via hydrophobic, ionic, steric, and/or Van der Waals forces (Butt et al., 2005). I observed

that DBeQ, but not bortezomib treatment, led to striking changes in key measures of adhesion, including energy dissipation. This suggests that protein within DBeQ-treated nodules was either less tightly bound to mineral or that the proteome of DBeQ-treated nodules differed from that in control or bortezomib-treated groups. Bone mineralisation is thought to occur when negatively charged proteins stabilise disordered mineral precursors, which later become more crystalline and grow (Nudelman et al., 2010), creating a nano-composite structure. This structure requires a precise protein composition to allow for correct formation. My observations suggest that in DBeQ-treated cultures, this balance may have been disrupted, preventing the formation of bone-like mineral with appropriate nanostructure. However, despite my quantitative insights, the effects of mild VCP/p97 impairment were subtle and not all adhesion interactions in DBeQ-treated groups displayed long-range, energy dissipative behaviours. Indeed, distributions revealed many measurements with short-range behaviour, similar to that in control and bortezomib-treated groups. My observations suggest that either a subset of cells secreted protein that produced aberrant interactions, or a subset of the secreted proteome was aberrant. There are several ways by which inhibition of VCP/p97 (or, possibly, functional impairment caused by VCP/p97 mutations) could alter the intra- and extracellular proteome. In very general terms, interference with the controlled degradation of the vast array of proteins degraded by the UPS in a VCP/p97-dependent manner may impact on any of the many cellular functions VCP/p97 governs - and thus interfere with the normal proteome. More specifically, impaired ERAD causes ER stress and triggers the UPR, which temporarily attenuates overall protein synthesis. Although I only observed subtle UPR effects, these may well have been sufficient to alter the synthesis and secretion of ECM proteins. This could be tested by proteomic analysis of both the

intracellular and secreted proteome. Indeed, our lab found that DBeQ and another VCP/p97 inhibitor triggered the UPR and inhibited protein synthesis in human cancer cell lines (Parzych et al., 2015). Our observations in cancer cells also showed that VCP/p97 inhibition affects intracellular amino acid levels, mTORC1 (mammalian target of rapamycin complex 1) signalling (a key regulator of protein synthesis), autophagy, and a number of metabolic pathways (Parzych et al., 2019). While it was not feasible to investigate whether and to what extent these effects also occur in hMSCs undergoing osteogenic differentiation, it appears likely that they play some role in the changes I observed.

TEM and SEM imaging suggested that DBeQ differentially affected the ultra- and micro-structure of the bone-like material created by hMSC compared to bortezomib treatment. Indeed, in DBeQ-treated samples, I observed amorphous rather than fibrillar proteinaceous matrix in the intercellular spaces. As apatite templates on fibrillar collagen in native bone, this observation lends further support to my AFM-based findings that mineral failed to strongly interact with appropriate protein to create a bone-like nano-structure. DDC-SEM images similarly suggested a lack of close association between less dense matrix and denser mineral in DBeQ-treated cultures. Moreover, high magnification secondary electron SEM images of DBeQ-treated cultures revealed needle-like mineral, akin to that observed in non-physiological precipitation reactions (Gebrehiwet et al., 2014). These observations suggest that in addition to an aberrant cell-mediated mineralisation process, the more crystalline mineral in DBeQ-treated cultures could have been formed in a pure physicochemical process, similar to that in pathological calcifications (Bertazzo et al., 2013).

Here, I describe a cell-derived, biomaterial-based *in vitro* model of bone formation whereby I used pharmacological inhibition of two key components of the intracellular protein degradation apparatus to induce mild cellular stress. I then identified a combination of relatively straightforward, widely available techniques from materials science that allowed me to quantitatively characterise changes to tissue biochemical composition, mechanical and adhesive properties, as well as observe morphological changes to their micro- and ultra-structure. This combination of materials science characterisation techniques is particularly amenable for analysing changes in bone-like tissue formation because neither AFM-based indentation, Raman spectroscopy nor the imaging techniques requires specific staining/labelling, which would require prior knowledge of specific targets of the inhibition. The limitation of this approach, of course, is that specific targets are not identified; however, as VCP/p97 and the proteasome inhibitors affect the proteome broadly, this approach may be superior. Moreover, as the techniques analyse the physical material created by cells, they can also detect potential post-translational changes in proteins, which may impact on cell/tissue function. These changes may be overlooked by standard proteomic techniques, for example. In time, techniques such as single cell proteomics that can analyse post-translation modifications to proteins may become widely available; however, until then, physical science-based characterisations may remain the gold standard.

As my *in vitro* experimental platform allows for the functional effects of proteostasis imbalances to be evaluated quantitatively, it may find use in high-throughput pre-clinical drug screening purposes (de la Puente et al., 2015), particularly to evaluate the functional effects on tissues of therapies designed to treat diseases stemming from perturbed proteostasis or for cancer therapies targeting protein degradation.

Given that multiple components of protein degradation pathways are being investigated as anti-cancer drug targets, my findings both highlight potential unwanted skeletal effects such approaches might bring, and also offer an early preclinical screening tool. Finally, cell-derived, ECM-based biomaterials akin to the mineralised nodules formed by my hMSC cultures are highly promising as potential scaffolds for tissue engineering (Evans et al., 2010, Yan et al., 2015). As these materials are secreted/arranged by tissue-specific cells, they are both composed of appropriate ECM and contain growth and other regulatory factors to direct seeded cells. However, tissue engineering approaches such as culture on 3D scaffolds can expose cells to stressors, such as reactive oxygen species, acidosis and hypoxia, all of which are known to perturb proteostasis and activate the unfolded protein response (Almanza et al., 2019). My finding that mildly perturbing proteostasis significantly impacts tissue properties suggests that maintaining proteostasis may be essential in forming ECM-based biomaterial scaffolds that replicate the native tissue.

5 Conclusion

Using a cell-derived model of bone formation in which I mildly perturb proteostasis, I have shown that a combination of techniques from materials science can quantitatively detect tissue effects that may explain some of the pathologies associated with VCP/p97-related diseases. This model may have important implications for studying VCP/p97-related diseases as there are no existing *in vivo* models that can replicate the subtle/intermittent proteostasis imbalances that characterise these diseases. Furthermore, this is the first *in vitro* model I am aware of that examines the effects of proteostasis imbalances on the mechanical, morphological and biochemical characteristics of tissues, as previous *in vitro* studies using pharmacological agents to perturb proteostasis have only carried out limited molecular analyses, which provide little insight into the tissue pathologies observed in patients. Moreover, whilst Raman spectroscopy, AFM and electron microscopy have been used to analyse tissues (Bertazzo et al., 2013, Tarnowski et al., 2003), including the material formed by cells *in vitro* (Evans et al., 2010, Gentleman et al., 2009, Volponi et al., 2015); to my knowledge this is the first study using these techniques to analyse tissue changes mediated by VCP/p97 and/or proteasome inhibition. Future work to further characterise the mechanisms underlying aberrant tissue formation linked to proteostasis impairment (i.e. caused by age, mutations, or drugs) should focus on effects on the intra- and extracellular proteome (using proteomic approaches), protein synthesis (such as semi-quantitative assessment of puromycinylation or ribosome profiling), and amino acid

levels. Given that these effects are tightly linked to cellular metabolism, metabolic studies (such as global metabolomic profiling) would also potentially be of value. Moreover, it would be useful to develop the approach I used into a semi-automated high-throughput assay so that larger numbers of drugs can be screened for potentially unanticipated effects on tissue biophysical effects.

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Appendices

Supplementary Table 1.

Primers used for gene expression analyses.

Proteostasis system

<i>ATF4</i>	F 5'-AGATGACCTGGAAACCATGC-3' R 5'-AGGGATCATGGCAACGTAAG-3'
<i>BIP (HSPA5)</i>	F 5'-GCCGTCCTATGTCGCCTTC-3' R 5'-TGGCGTCAAAGACCGTGTTTC-3'
<i>CHOP (DDIT3)</i>	F 5'-AGCAGAGGTCACAAGCACCT-3' R 5'-TTCATGCTTGGTGCAGATTC-3'
<i>GADD34 (PPP1R15A)</i>	F 5'-CTGAGCCCTGCCCCTTCCGAR-3' R 5'-GAAGCGCACCTTTCTGGCCT-3'
<i>P58IPK / P58 (DNAJC3)</i>	F 5'-TCCTGGTGGACCTGCAGTACG-3' R 5'-CTGCGAGTAATTTCTTCCCC-3'
<i>p97 (VCP)</i>	F 5'-CCCTGTGCCTGCTTCTTT-3' R 5'-GCTGCTCCCTTTCCACCA-3'
<i>TCF11 (NFE2L1)</i>	F 5'-GGAGGATTTGGGGGCTGTAG-3' R 5'-TCCTGTGCCAAAGGATGTC-3'
<i>TXNIP</i>	F 5'-TTCGGGTTTCTAGAAGATCAGG-3' R 5'-TGGATCCAGGAACGCTAAC-3'

Osteogenic genes

<i>RUNX2</i>	F 5'-ACAGTAGATGGACCTCGGGA-3' R 5'-ATACTGGGATGAGGAAATGC-3'
<i>ALPL</i>	F 5'-AACATCAGGGACATTGACGTG-3' R 5'-GTATCTCGGTTTGAAGCTCTTCC-3'
<i>IBSP</i>	F 5'-GGGCAGTAGTGACTCATCCG-3' R 5'-TCAGCCTCAGAGTCTTCATCTTC-3'
<i>SPARC</i>	F 5'-GATGGTGCAGAGGAAACCGA-3' R 5'-TTTGCAAGGCCCGATGTAGT-3'

Reference gene

<i>GAPDH</i>	F 5'-TCTGCTCCTCCTGTTGACA-3' R 5'-AAAGCAGCCCTGGTGACC-3'
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Primers were obtained from Integrated DNA Technologies.

Supplementary Table 2.

Statistical analyses of mineralisation quantification and colorimetric calcium content quantification in hMSC differentiated in commercially available Stembro® medium or classical osteogenic medium after 21 days of culture.

Mineralisation quantification	
Stembro® vs. Classical	$p = 0.5391$

Colorimetric calcium content quantification	
Stembro® vs. Classical	$p = 0.5391$

Statistical analyses were carried out using a non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test. $n = 3$, from 3 different donors.

Supplementary Table 3.

Statistical analyses of normalized viability of hMSCs undergoing osteogenic differentiation treated on days 0, 7, 14, and 21 with the indicated concentrations of DBEq for 48h evaluated by Alamar Blue assay ($n = 3$).

Concentration (μM)	DBEq (p)			
	day 0	day 7	day 14	day 21
0 vs. 1	>0.999	>0.999	>0.999	>0.999
0 vs. 1.25	>0.999	>0.999	>0.999	>0.999
0 vs. 2.5	>0.999	>0.999	>0.999	>0.999
0 vs. 5	>0.999	>0.999	>0.999	>0.999
0 vs. 7.5	>0.999	>0.999	>0.999	>0.999
0 vs. 10	>0.999	>0.999	>0.999	>0.999
0 vs. 12.5	>0.999	>0.999	>0.999	>0.999
0 vs. 25	>0.999	0.113	>0.999	>0.999
0 vs. 50	>0.999	0.025	0.421	>0.999
1 vs. 1.25	>0.999	>0.999	>0.999	>0.999
1 vs. 2.5	>0.999	>0.999	>0.999	>0.999
1 vs. 5	>0.999	>0.999	>0.999	>0.999
1 vs. 7.5	>0.999	>0.999	>0.999	>0.999
1 vs. 10	>0.999	>0.999	>0.999	>0.999
1 vs. 12.5	>0.999	>0.999	>0.999	>0.999
1 vs. 25	0.627	>0.999	>0.999	>0.999
1 vs. 50	0.181	0.325	>0.999	>0.999
1.25 vs. 2.5	>0.999	>0.999	>0.999	>0.999
1.25 vs. 5	>0.999	>0.999	>0.999	>0.999
1.25 vs. 7.5	>0.999	>0.999	>0.999	>0.999
1.25 vs. 10	>0.999	>0.999	>0.999	>0.999
1.25 vs. 12.5	>0.999	>0.999	>0.999	>0.999
1.25 vs. 25	0.712	0.452	0.209	>0.999
1.25 vs. 50	0.209	0.138	0.072	0.627
2.5 vs. 5	>0.999	>0.999	>0.999	>0.999
2.5 vs. 7.5	>0.999	>0.999	>0.999	>0.999
2.5 vs. 10	>0.999	>0.999	>0.999	>0.999
2.5 vs. 12.5	>0.999	>0.999	>0.999	>0.999
2.5 vs. 25	0.550	0.741	>0.999	>0.999
2.5 vs. 50	0.156	0.209	>0.999	0.712
5 vs. 7.5	>0.999	>0.999	>0.999	>0.999
5 vs. 10	0.550	>0.999	>0.999	>0.999
5 vs. 12.5	0.156	>0.999	>0.999	>0.999
5 vs. 25	0.038	>0.999	>0.999	>0.999
5 vs. 50	0.008	>0.999	>0.999	0.627
7.5 vs. 10	>0.999	>0.999	>0.999	>0.999
7.5 vs. 12.5	>0.999	>0.999	>0.999	>0.999
7.5 vs. 25	>0.999	>0.999	>0.999	>0.999
7.5 vs. 50	>0.999	>0.999	>0.999	>0.999
10 vs. 12.5	>0.999	>0.999	>0.999	>0.999
10 vs. 25	>0.999	>0.999	0.368	0.209
10 vs. 50	>0.999	>0.999	0.134	0.052
12.5 vs. 25	>0.999	>0.999	>0.999	0.368
12.5 vs. 50	>0.999	>0.999	0.627	0.099
25 vs. 50	>0.999	>0.999	>0.999	>0.999

Statistical analyses were carried out using a non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test.

Supplementary Table 4.

Statistical analyses of normalized viability of hMSCs undergoing osteogenic differentiation treated on days 0, 7, 14, and 21 with the indicated concentrations of bortezomib for 48h evaluated by Alamar Blue assay ($n = 3$).

	Bortezomib (p)			
Concentration (nM)	day 0	day 7	day 14	day 21
0 vs. 1	>0.999	>0.999	>0.999	>0.999
0 vs. 2.5	>0.999	>0.999	>0.999	>0.999
0 vs. 5	>0.999	>0.999	>0.999	>0.999
0 vs. 10	>0.999	>0.999	>0.999	>0.999
0 vs. 25	>0.999	>0.999	>0.999	>0.999
0 vs. 50	>0.999	>0.999	>0.999	>0.999
0 vs. 75	>0.999	>0.999	>0.999	>0.999
0 vs. 100	0.866	>0.999	>0.999	>0.999
0 vs. 250	>0.999	>0.999	>0.999	>0.999
0 vs. 500	0.695	>0.999	>0.999	>0.999
0 vs. 1000	>0.999	>0.999	>0.999	>0.999
1 vs. 2.5	>0.999	>0.999	>0.999	>0.999
1 vs. 5	>0.999	>0.999	>0.999	>0.999
1 vs. 10	>0.999	>0.999	>0.999	>0.999
1 vs. 25	>0.999	>0.999	>0.999	>0.999
1 vs. 50	>0.999	>0.999	>0.999	>0.999
1 vs. 75	>0.999	>0.999	>0.999	>0.999
1 vs. 100	>0.999	>0.999	>0.999	>0.999
1 vs. 250	>0.999	>0.999	>0.999	>0.999
1 vs. 500	>0.999	>0.999	>0.999	>0.999
1 vs. 1000	>0.999	>0.999	>0.999	>0.999
2.5 vs. 5	>0.999	>0.999	>0.999	>0.999
2.5 vs. 10	>0.999	>0.999	>0.999	>0.999
2.5 vs. 25	>0.999	>0.999	>0.999	>0.999
2.5 vs. 50	>0.999	>0.999	>0.999	>0.999
2.5 vs. 75	>0.999	>0.999	>0.999	>0.999
2.5 vs. 100	>0.999	>0.999	>0.999	>0.999
2.5 vs. 250	>0.999	>0.999	>0.999	>0.999
2.5 vs. 500	>0.999	>0.999	>0.999	>0.999
2.5 vs. 1000	>0.999	>0.999	>0.999	>0.999
5 vs. 10	>0.999	>0.999	>0.999	>0.999

5 vs. 25	>0.999	>0.999	>0.999	>0.999
5 vs. 50	>0.999	>0.999	>0.999	>0.999
5 vs. 75	>0.999	>0.999	>0.999	>0.999
5 vs. 100	>0.999	>0.999	>0.999	>0.999
5 vs. 250	>0.999	>0.999	>0.999	>0.999
5 vs. 500	>0.999	>0.999	>0.999	>0.999
5 vs. 1000	>0.999	>0.999	>0.999	>0.999
10 vs. 25	>0.999	>0.999	>0.999	>0.999
10 vs. 50	>0.999	>0.999	>0.999	>0.999
10 vs. 75	>0.999	>0.999	>0.999	>0.999
10 vs. 100	>0.999	>0.999	>0.999	>0.999
10 vs. 250	>0.999	>0.999	>0.999	0.494
10 vs. 500	>0.999	>0.999	>0.999	>0.999
10 vs. 1000	>0.999	>0.999	>0.999	>0.999
25 vs. 50	>0.999	>0.999	>0.999	>0.999
25 vs. 75	>0.999	>0.999	>0.999	>0.999
25 vs. 100	>0.999	>0.999	>0.999	>0.999
25 vs. 250	>0.999	>0.999	>0.999	0.695
25 vs. 500	>0.999	>0.999	>0.999	>0.999
25 vs. 1000	>0.999	>0.999	>0.999	>0.999
50 vs. 75	>0.999	>0.999	>0.999	>0.999
50 vs. 100	>0.999	>0.999	>0.999	>0.999
50 vs. 250	>0.999	>0.999	>0.999	>0.999
50 vs. 500	>0.999	>0.999	>0.999	>0.999
50 vs. 1000	>0.999	>0.999	>0.999	>0.999
75 vs. 100	>0.999	>0.999	>0.999	>0.999
75 vs. 250	>0.999	>0.999	>0.999	0.494
75 vs. 500	>0.999	>0.999	>0.999	>0.999
75 vs. 1000	>0.999	>0.999	>0.999	>0.999
100 vs. 250	>0.999	>0.999	>0.999	>0.999
100 vs. 500	>0.999	>0.999	>0.999	>0.999
100 vs. 1000	>0.999	>0.999	>0.999	>0.999
250 vs. 500	>0.999	>0.999	>0.999	>0.999
250 vs. 1000	>0.999	>0.999	>0.999	>0.999
500 vs. 1000	>0.999	>0.999	>0.999	>0.999

Statistical analyses were carried out using a non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test.