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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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ABSTRACT

Cellular function depends on the maintenance of protein homeostasis (proteostasis) by regulated protein degradation. Chronic dysregulation of proteostasis is associated with neurodegenerative and age-related diseases, and drugs targeting components of the protein degradation apparatus are increasingly used in cancer therapies. However, as chronic imbalances rather than loss of function mediate their pathogenesis, research models that allow for the study of the complex effects of drugs on tissue properties in proteostasis-associated diseases are almost completely lacking. Here, to determine the functional effects of impaired proteostatic fine-tuning, we applied a combination of materials science characterisation techniques to a cell-derived, *in vitro* model of bone-like tissue formation in which we pharmacologically perturbed protein degradation. We show that low-level inhibition of VCP/p97 and the proteasome, two major components of the 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 failed 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 utility 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. Our 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.

1. Introduction

Accurate and stable maintenance of cellular protein homeostasis (proteostasis) is critical for tissue integrity and has been linked to longevity [1–4]. Perturbed proteostasis contributes to the pathogenesis of a myriad of predominantly age-related diseases ranging from neurodegenerative disorders to diabetes and cancer [5–7]. An elaborate network of mechanisms constantly monitors and fine-tunes the intracellular proteome [8,9]. The controlled degradation of proteins that are dysfunctional, damaged, or no longer needed is central to this

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process and is primarily executed by the ubiquitin-proteasome system (UPS). The UPS recognises proteins that have been earmarked for degradation by the addition of polyubiquitin chains, and degrades them in the 26S proteasome, thereby regulating multiple cellular functions, including stem cell fate [10–13]. Small molecule inhibitors of the proteasome are widely used in the treatment of multiple myeloma, and pharmacological targeting of other UPS components is a major area of anti-cancer research [14].

The ATPase VCP/p97 plays a central role in the UPS by extracting ubiquitinated proteins from cellular structures and delivering them to the proteasome [15–19]. Drugs targeting VCP/p97 have therapeutic potential as anti-cancer agents and for the treatment of epilepsy linked to GABAA receptor mutations [20–22]. VCP/p97 mutations have been associated with a 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 [23–28]. In short, VCP/p97 is essential for cellular proteostasis and maintains skeletal and neurological health [29].

Although the pathogenesis of VCP/p97-related diseases remains to be established, their associated cellular dysfunction has been linked to defective proteostatic fine-tuning [29–31]. This putative mechanism is compatible with the notion that relatively minor but chronic or intermittent imbalances in proteostasis contribute to many age-related diseases [4,29]. However, perturbations in fine-tuning that result in chronic or intermittent imbalances in intracellular proteostasis are extremely challenging to replicate in *in vitro* and particularly in *in vivo* models. Indeed, the relative difficulty of establishing a research model when impairment rather than loss of function mediates complex tissue pathologies has hampered research efforts to identify the pathogenesis of VCP/p97-related diseases. Moreover, there are currently no robust experimental paradigms to study the functional effects of intracellular proteostasis imbalance or test potential therapeutic compounds that modulate proteostasis. In short, a research platform that could mimic the functional tissue effects of chronic or intermittent proteostasis imbalances could be invaluable in both exploring disease mechanisms and screening for drug effects.

The bone-like material that can be formed by osteogenic cells *in vitro* constitutes a highly informative model system to study how impaired intracellular proteostasis might impact functional tissue properties. As mesenchymal stromal/stem cells (MSC) differentiate down the osteogenic lineage and synthesise large amounts of extracellular matrix (ECM), they become highly dependent on mechanisms which control proteostasis [32–34]. This secreted proteinaceous matrix is then progressively mineralised by poorly crystalline carbonated apatite, producing a bone-like nano-composite structure in a highly controlled process such that even small perturbations to the composition of either the proteinaceous or mineral phases can significantly impact bone quality [35–37], providing a read-out of proteostasis imbalance. This model is also of direct clinical relevance because the pathogenesis of VCP/p97-related bone disease is incompletely understood; and whilst proteasome inhibitors purportedly stimulate bone regeneration in myeloma patients, the effects of drugs targeting VCP/p97 on bone have not been established [38–40]. Moreover, cell-derived, ECM-based materials have been proposed as promising scaffolds to direct SC differentiation in tissue engineering applications [41,42]. Therefore, insight into how proteostasis imbalances may impact these biomaterials' functional properties may be important for creating scaffolds that appropriately mimic native tissues.

To understand how impaired proteostatic fine-tuning functionally affected tissue, we created an *in vitro* model using intermittent low-level proteasome or VCP/p97 inhibition in human MSC (hMSC) as they differentiated into osteoblasts and formed a cell-derived, bone-like material (Supplementary Fig. 1). We show that low-level inhibition of VCP/p97 and the proteasome differentially affect the bone-like material that hMSC form *in vitro*. Indeed, whilst proteasome inhibition subtly promotes the formation of a material akin to native bone, Raman spectroscopic, atomic force microscopy (AFM)-based indentation, and electron microscopy imaging suggest that VCP/p97 inhibition results in a material that is softer and less proteinaceous, but whose mineral appears to be more crystalline and morphologically aberrant. These observations suggest that mild VCP/p97 impairment in hMSC undergoing osteogenic differentiation may alter tissue physical properties in a way that could explain some of the pathologies associated VCP/p97-related diseases, including changes in bone mechanical properties [43]. Our results highlight the utility of applying materials science approaches to challenges that biological techniques cannot yet address. They may also provide the basis for *in vitro* platforms that would allow for the functional effects of proteostasis imbalances to be evaluated quantitatively in a model that could be particularly relevant for high-throughput pre-clinical drug screening purposes. Finally, our findings suggest that the fabrication of biomaterial scaffolds that utilise cell-derived matrices may need to consider the effects of proteostasis in order to properly match scaffold properties to those of the native tissue.

2. Results

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

To develop an *in vitro* model of proteostasis imbalance, we first aimed to determine if we could mildly perturb proteostasis in hMSC undergoing osteogenic differentiation. Genetic approaches to deplete VCP/p97 or the proteasome are not suitable to study the effects of mild functional impairments [20,44]. Therefore, we took a pharmacological approach and treated hMSC with either the well-characterised and highly selective VCP/p97 inhibitor, DBeQ [45–48], or the first-in-class clinical proteasome inhibitor, bortezomib [49]. To define inhibitor concentrations that would induce mild functional impairment without overt toxic effects, we initially determined IC_{50} values for viability. We found that osteogenic differentiation increased the IC_{50} for DBeQ (as determined by cellular metabolic activity) from 7.5 μM in undifferentiated hMSC to 22 μM in their differentiated progeny (Fig. 1a). For comparison, bortezomib, which effectively kills multiple myeloma cells *in vitro* at concentrations of 10–20 nM [47] (Supplementary Fig. 2), did not reduce viability of differentiating hMSC at concentrations up to 1000 nM (Fig. 1a). Next, we aimed to determine the degree of proteotoxic stress caused by a concentration of DBeQ that did not affect viability (5 μM) at any stage of *in vitro* differentiation compared to a clinically relevant concentration of bortezomib (20 nM) by quantifying the expression of a panel of genes encoding proteins with key roles in proteostasis. DBeQ and bortezomib both induced a very mild proteotoxic stress response, as determined by low-level changes in proteostasis gene mRNA levels that were largely non-significant (Fig. 1b and Supplementary Table 1). For comparison, the protein glycosylation inhibitor tunicamycin, which causes protein misfolding in the endoplasmic reticulum, resulted in more pronounced changes in proteostasis gene mRNAs when given at a nonlethal dose (Fig. 1b and Supplementary Table 1). However, immunoblotting for ubiquitinated proteins confirmed that bortezomib and DBeQ perturbed the UPS, with a clear increase in the level of ubiquitinated proteins in cells treated with bortezomib, while DBeQ had a minor effect (Fig. 1c and Supplementary Fig. 3). These observations demonstrate that by fine-tuning an appropriate dose in differentiating hMSC, DBeQ and bortezomib can impair intracellular proteostasis and trigger mild proteotoxic stress that is not acutely toxic to cells.

2.2. 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. To study the effects of VCP/p97 and proteasome inhibition on this process, we first used