Chapter 6

Final Discussion

6.1 Overall summary of research findings

The BM microenvironment contains a mixture of a number of different cell types which, through their interactions, provide an ideal environment for cancerous cells to grow. This complex environment includes, osteogenic cells, endothelial cells, adipocytes, MSC, macrophages and haematopoietic cells (Jurczyszyn et al., 2015; Balakumaran et al., 2010; Fernandez et al., 2008) and serves as a safe haven not only for normal healthy cells but also for malignant cells such as MM cells (Yang and Lin, 2015; Anderson, 2007; Podar et al., 2007). The overall aims of this thesis were to develop a relevant model for the co-culture of MSC/HS5 and MM cells (chapter 3), to investigate the effects of chemotherapy on the functionality of MSC/HS5 using the developed in vitro model (chapter 4) and to assess the potential of these agents to cause genotoxicity within the co-culture model (chapter 5). Here, how these interactions affect MSC and MM cells with and without chemotherapy has been investigated.

It is practically impossible to reproduce the cancer microenvironment using a cancer cell line in isolation using an in vitro analysis. Therefore, to address the aims of this study, a suitable in vitro co-culture model of MM within the BM was developed for investigating the effects of chemotherapy exposure on physical characteristics, functionality and genotoxicity. It was important to choose an appropriate cell source that would give an accurate representation of the BM microenvironment in MM. In developing this MM co-culture model a number of cells were used; primary BM-MSC obtained from patients who had undergone a total hip replacement, the stromal cell line HS5 and the MM cell lines U266B1 and RPMI 8226. Each of these cells have differences in metabolism and require particular nutrients for their growth. In order to achieve good experimental reproducibility, cell types need to be cultured in the same cell culture medium.
Cellular proliferation and morphology was monitored in a range of culture media (DMEM/LG, DMEM/HG, DMEM/F12 and RPMI 1640) to select the medium that best sustains all the cell populations. It was noted that DMEM/F12 culture medium provided suitable support to the proliferation of each these cells. The morphological characteristics of primary MSC and HS5 were unchanged in DMEM/F12, with both cell types exhibiting a similar fibroblast-like morphology. Subsequently DMEM/F12 was used for all cell culture studies.

Co-cultures could be carried out potentially at any volume range and cell density which can be important factors when establishing biomimetic in vitro models (Goers et al., 2014). Typically, published research specifies the culture techniques used for cells without further specification of seeding density. Here MSC and HS5 cell density was confirmed by monitoring the cells’ proliferation in 12 well plates over the course of 1 week. Seeding MSC at low density has been documented to improve proliferation whereas a high density can increase the risk of cell death due to nutrient limitation (Colter et al., 2001). Here it was important that there was enough space within the plate to allow for MSC/HS5 cell growth over a 7 day period yet also sufficient cells for analysis at certain time points.

The direct co-culture of MM cells with stromal cells proved difficult due to problems associated with separation of the cells. Cells would need to be separated for a number of assays. After a number of attempts to remove the MM cells from the co-culture with MSC and HS5 it was clear that MM cells could not be fully detached, thereby contaminating the adherent cell culture. Therefore transwell inserts were used to separate the cells. However this method is limited as it relies on the release of soluble factors to provide interactions. Many studies have demonstrated that direct interactions between MM cells and MSC lead to chemotherapy resistance and increased growth and survival of MM cells (Hao et al., 2011b; Nefedova et al., 2003). However, the literature documents less well, how to recover cells from a direct co-culture. Therefore, development of a method whereby MSC would be in closer proximity to MM cells and provide possible contact with a suitable method for detachment was attempted. Here MSC demonstrated the ability to adhere to the underside
of a transwell insert and potentially interact with MM cells on the other side. SEM revealed that U266B1 cells had projections that were small enough to pass through the pores of the insert, with MSC on the other side. HS5 stromal cells, however, were unable to remain attached to the insert and this method was not investigated further. Both methods can be used, depending on the special experimental requirements.

Cell culture studies are useful tools that enable biological questions to be investigated. Both these co-culture models allow testing of a number of other areas of the BM microenvironment and bone cancer hypotheses. More broadly, many researchers would likely increase the reproducibility of their research by first identifying suitable medium for the co-culture of different cell types.

Whilst this model provides a basis to study the interactions of these cells, it is not without its limitations. The model developed in this study has been conducted under 2D conditions. In order to better understand the relationships of the BM microenvironment and malignant cells, a 3D culture system may be required. Since cells of the BM microenvironment are inherently 3D, the aforementioned 2D system does not fully take into account the in vivo environment of these cells. Recently scaffolds and hydrogels have been developed that allow the cells to maintain their 3D structure (Knight and Przyborski, 2015; Tibbitt and Anseth, 2009). However, further development of these 3D cell culture materials is required to assure reproducibility, high throughput analysis, compatible readout techniques and automation in order to establish a uniform 3D culture model (Edmondson et al., 2014). Until then, the traditional 2D cell culture remains the most reliable in vitro testing system for the analysis of disease and drugs.

Following establishment of a model, chemotherapeutic agents currently used in the treatment of MM were used to investigate their effects on both MSC/HS5 and U266B1 cells cultured independently, in non-contact co-culture and following exposure to a chemotherapeutic agent to one cell compartment before culturing with unexposed cells (bystander effect). Functionality and genotoxicity methods were used to ascertain the effects of chemotherapy within the in vitro model.
Damage to MSC caused by chemotherapeutic agents may have a possible involvement in the progression of MM, and also have a role in poor haematopoietic engraftment after ASCT (Spitzer, 2015; Reagan and Ghobrial, 2012). The extent of damage to MSC while in co-culture with an MM cell line and exposed to chemotherapy was previously unknown. Here this study has demonstrated chemotherapy induced changes in MSC and HS5 proliferation, physical characteristics and differentiation. Furthermore, significant findings were also discovered following co-culture with U266B1 cells when untreated, suggesting the presence of hematological disease can also effect the BM.

Cytotoxic effects measured by trypan blue varied amongst chemotherapeutic agents. Significant effects were observed on primary MSC, HS5 stromal cells and U266B1 cells following exposure to melphalan and proteasome inhibitors when cells were cultured independently. When in co-culture, U266B1 cells had improved cell growth and viability after exposure to melphalan compared to independent cultures. Primary MSC and HS5 cells, however, experienced a further decrease in their proliferation and viability. The same findings were also reported with bortezomib and carfilzomib, whereby HS5 cells were further impaired as a result of a co-culture with U266B1, with U266B1 cells gaining some protection with improved cell numbers and viability. Immunomodulatory agents, thalidomide and lenalidomide did not significantly affect cell numbers or viability when cells were cultured independently or in co-culture. These findings demonstrate a tropism of MSC and HS5 towards the MM cell line U266B1. Furthermore this research also reveals that cell-cell contact is not essential to provide protection of both the BM and MM cells against chemotherapy which had previously been reported (Hao et al., 2011; Shain and Dalton, 2001).

This study, for the first time, describes the morphology of HS5 cells following exposure to chemotherapeutic agents. MSC morphology has been documented to become altered following exposure to chemotherapy (May et al., 2010). A study by Kemp and colleagues (2010) reported a loss of characteristic fibroblast morphology of primary MSC with the appearance of elongated projections from the cell membrane following treatment with cyclophosphamide in vitro and in vivo. This study has demonstrated similar observations following exposure to
immunomodulatory agents and proteasome inhibitors in vitro using the cell line HS5.

In particular, HS5 cells treated with proteasome inhibitors carfilzomib and bortezomib observed an increase in cells ‘floating’ in the culture medium with adherent cells becoming aggregated compared to the uniform fibroblast-like appearance that were observed in untreated cultures. The immunomodulatory agent lenalidomide had a more pronounced effect on morphology compared to its parent drug thalidomide, with an increase in elongated cells and aggregation. Interestingly, melphalan did not produce significant changes in HS5 morphology when administered to independent HS5 cultures. However as with each of these agents, HS5 morphology was further exacerbated when in co-culture with U266B1 cells. As cells respond to microenvironment cues, they can adopt different shapes, generate traction stress and produce mechanical forces that can be transmitted to neighbouring cells (Li et al., 2011). These disruptions in HS5 morphology may have significant implications on their ability to carry out normal functions such as support of haematopoiesis and differentiation as well as their survival and proliferation.

MSC differentiation is critical for bone homeostasis (Carroll et al., 2012). MM is known to block osteogenic differentiation of MSC preventing further bone production (Fu et al., 2010) with recent research indicating adipocytes may accelerate or support MM (Falank et al., 2016). The results of chapter 4 indicated that HS-5 stromal cell osteogenic and adipogenic differentiation was reduced as a result of a non-contact co-culture with U266B1 cells. Additionally chemotherapy treatment was found to further disrupt cell differentiation. This could suggest growth factors within the medium necessary for HS5 differentiation were taken up by the U266B1 cells or the effect of co-culture increases the release of signalling molecules by apoptotic MM cells and lead to additional stress signals that enhance the effect of the chemotherapeutic agents (Berenstein et al., 2015).

MSC are commonly known to express the cell-surface marker CD105 and CD73 (Maleki et al., 2014; Dominici et al., 2006) and are recognized to play important roles in the immune response. In this report the expression of these
markers along with CD45, CD34 and CD14, of which these cells are known to lack expression, was evaluated in HS-5 following exposure to chemotherapy. The significantly increased expression of CD105 following melphalan exposure could provide possible justification to the reduced osteogenic and adipogenic seen in this study, since CD105 has previously been closely associated with the osteogenic and adipogenic potential of stromal cells (Anderson et al., 2013; Levi et al., 2011). Both melphalan and bortezomib were found to increase the expression of CD105 on HS5 cells. With CD73 documented to be involved in drug resistance (Gao et al., 2014), increased expression of this surface antigen following chemotherapy could be related to the drug resistance that is seen in some patients with MM. Moreover increased expression of these CD markers may effect interactions with MM cells and future studies should investigate the levels of expression of these markers when in co-culture. Further studies of the multiple downstream signalling pathways involved in both of these CD markers will be required to confirm their role in relation to chemotherapeutic treatment and MM.

A number of cytokines have been described to be influential in the pathogenesis of MM (Lee et al., 2004; Gupta et al., 2001; Otsuki et al., 2000) with IL-6 playing a significant role as a growth and survival factor of MM cells (Scheller and Rose-John, 2006; Gado et al., 2000). In the current study IL-6 was measured following the development of an in-house sandwich ELISA which allowed the detection of IL-6 in the supernatants of independent cultures and co-cultures. Analysis of the cytokine IL-6 investigated in this study allows us to draw some conclusions.

This report has shown that following exposure to a variety of chemotherapeutic agents IL-6 concentrations are reduced in cell supernatants in both alone and co-cultures. For the first time a significantly higher concentration of IL-6 was demonstrated in primary MSC compared to the stromal cell line HS5 when cultured alone and untreated. In agreement with previous studies (Bam et al., 2015; Giuliani et al., 2004), it was confirmed that mean IL-6 concentrations are significantly elevated in a co-culture of MSC/HS5 and MM cells. DNA damaging effects of melphalan have been documented to be reduced in the presence of
IL-6 (Efferth et al., 2001). With the high levels of IL-6 that were seen in co-cultures of MSC and U266B1 this could explain the reduced DNA damaging effects that were seen in co-cultures exposed to melphalan. Immunomodulatory agents and proteasome inhibitors investigated here are not known to directly inhibit the secretion of IL-6. However this study revealed that each of these agents significantly reduced the concentration of IL-6 in U266B1 cells when cultured alone. When administered to the co-culture of HS5 and U266B1, lenalidomide had the most pronounced effect with both bortezomib and carfilzomib also significantly reducing the concentration of IL-6.

Elevated levels of IL-6 in co-culture experiments concur with the increase in MM cell numbers that were seen when compared to independent culture. This finding agreed with previous studies that have demonstrated that IL-6 provides a growth advantage to MM cells along with a resistance to drug-induced apoptosis (Brown et al., 2012; Frassanito et al., 2001). Additionally, increased levels of IL-6 have previously been demonstrated to reduce adipogenic differentiation of MSC (Pricola et al., 2009). Therefore the finding of increased concentrations of IL-6 in co-culture may explain the inhibition of adipogenic differentiation of HS5 cells when in co-culture with U266B1.

With reports of increased IL-6 concentrations in MM patients compared to healthy controls (Singh et al., 2015), this cytokine could potentially be a good bio-marker for this disease. Previous studies have highlighted significantly elevated levels of IL-6 in MM patients compared to its often precursor, monoclonal gammopathy of undetermined significance (MGUS) (Nachbaur et al., 1991) and thus IL-6 may be a suitable cytokine in diagnosing MM. However, levels of IL-6 in serum and plasma are often raised in a number of conditions, so existing disease criteria would also be required alongside the measurement of this cytokine.

In chapter 5 of this thesis, several methods were utilised to measure the genotoxic effects of chemotherapy within the co-culture model. When investigating levels of DNA damage using the comet assay, immunomodulatory agents and proteasome inhibitors did not reveal significant levels of DNA damage at any time point in either U266B1 or HS5 cells. Melphalan was the
only agent that provided significant results in MSC, HS5 and U266B1 cells. DNA crosslinks were evident at 16 hrs in U266B1 cells following melphalan exposure, as evidenced by the reduction in the intensity of the comet tail with the increase in tail intensities at 48 and 72 hrs. Interestingly, percentage DNA in the comet tail was lower at each time point when in co-culture with U266B1 suggesting that MSC and HS5 protected the U266B1 cell from DNA damage. 

H$_2$O$_2$ was used to create a fixed amount of DNA damage in the form of strand breaks and the results clearly showed that increased DNA damage occurred following exposure to H$_2$O$_2$ in all samples. Surprisingly MSC and HS5 stromal cells were not significantly affected as a result of melphalan exposure as measured by comet assay. To further investigate whether these agents induce genotoxic events in these cell lines the MN assay was used.

Results observed found that melphalan induced MN in both primary MSC and HS5 stromal cells in a time dependant manner. MN were found to decrease within U266B1 and MSC/HS5 cells when co-cultured together conferring protection to both cell types from the genotoxic effects of melphalan. Formation of MN was not significantly observed when cells were exposed to each of the other agents, either alone or in co-culture.

Finally, the study identified a significant number of multi-nucleated HS5 and U266B1 cells following exposure to thalidomide and lenalidomide. However, the precise reason for the rise in multi-nucleation following exposure to these agents remains elusive. Furthermore, multi-nucleated cells were reduced when agents were administered to the cells in co-culture. Additionally, multi-nucleation was significantly higher in cell synchronised TK6 cells following exposure to thalidomide and lenalidomide. The rise in multinucleated cells may provide a possible explanation for the peripheral neuropathy seen in some patients who have been administered these agents. PN has been documented to affect quality of life and limits the ability to continue effective treatment and has been documented in patients receiving thalidomide and lenalidomide. (Tacchetti et al., 2014; Martin, 2013; Argyriou et al., 2008), yet the aetiology of this toxicity remains unclear. Although it is believed that these agents may have
direct effects on the dorsal root ganglia leading to neuronal degeneration (Grisold et al 2012).

A study by Inatsuki and colleagues (2005) documented that an analogue of thalidomide perturbed microtubule dynamics by inhibiting the formation of microtubules in leukaemic cells. The ability of a thalidomide analogue to disrupt these microtubules along with a rise in multi-nucleation seen in this study could have profound consequences on understanding the mechanism of actions of these agents. Moreover, as these microtubules consist of alpha and beta tubulin dimers that are present in peripheral nerve fibres (Alberts et al., 2002) it is reasonable to assume that these agents lead to PN via disruption of microtubules within peripheral nerve fibres.

The MN assay is attractive due to its simplicity; however results from the HUMN (HUman MicronNucleus) project, found that there are large differences between results of different scorers and laboratories, in the interpretation of scoring criteria (Fenech et al., 2003). This study followed OECD guideline recommendations for the in vitro micronucleus assay. However future work should consider automated procedures such as flow cytometry to eliminate variation in visual scoring as well as human error.

Overall, development of a suitable MM in vitro model has enabled confirmation of the original hypothesis that MSC are damaged by chemotherapy treatment and exposure to MM cells. Furthermore, this study has demonstrated that damage to MSC is manifested through changes in functionality with evidence of genotoxic damage. Chemotherapeutic damage to stromal cells has been shown to increase as a result of co-culture with MM cells, with MM cells often protected.

6.2 Limitations of the study

It is important to mention that this study has not been without its limitations which may have had an impact on the findings. It has predominantly been undertaken with the use of one stromal cell line and one MM cell line. To further corroborate this data it is vital that other MM cell lines such as RPMI 8226
(Nilsson et al., 1968) as well as primary MSC from healthy patients and patients suffering with MM be used to complement this work.

As mentioned previously, this study has looked at the effects of chemotherapy using a non-contact model. In order to increase the clinical relevance of the study co-culture of the cells in direct contact should be investigated. However future models would still require methods for fully separating cells for analysis which proved difficult in this study.

As not all differentiation procedures were carried out, it would also be useful to investigate any effects on chondrogenic differentiation on MSC/HS5. Furthermore, due to issues with the flow cytometry it was not possible to analyse samples that had been co-cultured with U266B1 cells. Further optimisation of this technique will allow this to be investigated. With additional time, further investigations (outlined below) could be carried out, as this this study has generated a number of natural avenues of further work.

6.3 Future considerations

The results presented in this study provide some quite unexpected findings and an interesting basis for further experiments. With further time and funding, there is much scope to expand knowledge in this area. The results described here provide only a brief insight into the interactions of MM and its BM microenvironment.

As mentioned in chapter 3, MSC seeded on the underside of the insert may provide contact between the MM cells and MSC thus providing a suitable contact model for future analysis. With suitable ethical approval further work should investigate the effects of chemotherapy and MM on primary cells seeded in this manner. Additional research could also investigate the possible role of tumour load on MSC by increasing the density of MM cells within the in vitro model. Many chemotherapeutic regimens combine chemotherapy and such combinations should also be investigated within this in vitro model of MM. Furthermore, with a range of cytokines implicated in MM, future work should determine levels of these as well as investigating, levels of intracellular cytokines within the model.
Further investigation of the possible disruption to microtubules following treatment with immunomodulatory agents and proteasome inhibitors will be needed. To further support findings, fluorescently labelled tubulin dimers and live imaging could be used to observe spindle formation. Immunofluorescent antibodies used to stain the centrioles, which have an important role in ensuring correct chromosome segregation, will help determine if they separate abnormally with exposure to immunomodulatory agents. Moreover, investigation into the levels of ROS present within the model may further aid in understanding the mechanism of action of these agents. All of this combined will support the hypothesis and fully elucidate the role of these drugs in regard to therapy related PN and carcinogenesis. A better understanding of these novel agents will help to understand the possible side effects of these treatments and also improve the treatment regimen for patients with MM.

### 6.4 Thesis summary and conclusion

This study set out to achieve three key aims. In doing so, it has generated a comprehensive picture of the effects of MM cells on BM-MSC and vice versa with chemotherapy. It has been well documented that MM cells become protected from, and gain resistance to, chemotherapy, as a result of interactions with the BM microenvironment (Abdi et al., 2013; Basak et al., 2009). However, until now, few studies have reported the effects on BM-MSC as a result of these interactions and chemotherapy exposure. Whilst it has already been documented that MSC become damaged by chemotherapy exposure *in vitro* (May et al., 2010; Kemp et al., 2010) this study has revealed that MSC also become impaired as a result of a non-contact co-culture with MM cells *in vitro*. Furthermore damage has also been shown to be exacerbated by chemotherapy in an *in vitro* co-culture model with genotoxic effects of some agents demonstrated.

It was hypothesised that BM-MSC altruistically protect MM cells following chemotherapy exposure in an *in vitro* model of MM. Data gained within this study would strongly suggest BM-MSC are further impaired by the effects of chemotherapy and by MM cells, with both cytotoxic and genotoxic effects seen. Damage to MSC had not previously been defined following chemotherapy. Here
damage to the stromal cell line HS5 in a co-culture with MM cells has been demonstrated in a number of ways within this investigation. Reduction in cell numbers and viability, alteration in cellular morphology, inability to differentiate, expression of surface antigens, changes in cytokine expression, levels of DNA damage and presence of multi-nucleation have all been measured. The findings reported here also correlate with that of others who have reported an improvement in the growth of cancerous cells in the presence of MSC (Ljubic et al., 2013; Huang et al., 2013). This supportive quality of MSC/HS5 has often been at detriment to itself and thus may be described as altruistic to MM cells. However when studying the genotoxic effects of agents using the micronucleus assay it was noted that a co-culture protected both cell types from observed genotoxic events such as multi-nucleation compared to when cultured independently. Furthermore, a bystander effect was also documented in U266B1 cells that were never exposed to thalidomide directly.

Research into the interactions of MM and BM-MSC and vice versa has improved our understanding of the pathophysiology of the disease while also recognizing the implications for BM-MSC. Due to the vital role of MSC within the BM, any disruptions in their ability to undertake normal biological functions by either disease or chemotherapy could have long term consequences on the overall health of an individual.

This research provides further understanding of how these interactions affect MSC functionality and also protect from genotoxic damage. However, this picture is by no means complete, with this study providing a platform for additional research to enable further understanding of the mechanisms of how these drugs effect the interactions of MSC and MM which could lead to improved clinical applications of these agents. Whilst chemotherapy remains the most effective treatment for MM, results from this work indicate some of the side-effects of this therapy within the BM microenvironment. Ultimately reducing unintentional damage of cells of the BM microenvironment such as MSC could improve the prognosis for patients with MM and other haematological malignancies.