Chapter 2

Materials and Methods

2.1 General Methods

The procedures detailed in this thesis were conducted in accordance with both the University of the West of England Research Ethics Committee (UREC) and National Research Ethics Service (NRES), where appropriate. All clinical samples were obtained with prior informed consent from the donor and coded for anonymity.

2.1.1 Materials

All reagents were purchased from Sigma Aldrich (Dorset, UK) unless otherwise stated.

2.1.2 Samples

All sample preparation was carried out in a class II biological safety cabinet and all procedures were performed using sterile techniques to prevent contamination of the samples.

2.1.3 MSC sample collection

BM samples were processed previously by Dr Ruth Morse and Dr Craig Donaldson. The procedure of BM collection is briefly highlighted here. BM samples were obtained by an orthopaedic surgeon at the Avon Orthopaedic Centre (AOC), Southmead Hospital, Bristol, with full and informed patient consent along with NRES approval. BM was taken at the time of total hip replacement and was removed from the femoral shaft during surgery to make space for the prosthetic joint. Marrow scrapings were then collected by medical staff and placed into sterile 25 ml tubes containing 100 I.U. heparin and 4 mls DMEM/LG. Patients with any history of malignancy, immune disorders or rheumatoid arthritis were excluded from the study. Patients who had received

any drugs known to be associated with myelosuppression or BM failure were excluded from BM collections.

2.1.4 Cell lines

The MM cell lines U266B1 and RPMI 8226, from European Collection of Cell Culture (ECACC) along with the BM/stroma HS5 cell line from American Type Culture Collection (ATCC) were used. The lymphoblast cell line TK6 was donated by Dr Ann Doherty (AstraZeneca, UK) (Table 2.1).

2.1.5 Resistant Cells

Resistant MM cells were developed by a previous PhD student (Dr Mohammed Salehan).

U266B1 cells were exposed to gradually increasing doses of melphalan. The initial dose of melphalan was 0.5 µM and was added to the culture whenever the medium was changed. The dose of melphalan was escalated to a maximum dose of 5.0 µM over a year of culture. U266B1 cells that were previously cryopreserved at this concentration were selected and maintained by the addition of melphalan (5.0 µM) at each medium change.

Table 2.1 – The cell lines analysed in this study.

Cell Line Name	Type of Cell	Seeding Density	Proliferation Time (hrs)	Obtained From
RPMI 8226	Myeloma Cell	$3x10^5$ ml	50-60	ECACC
U266B1	Myeloma Cell	$3x10^5$ ml	50-60	ECACC
HS5	Stromal Cell	$2x10^5$ cm^2	50-60	ATCC
TK6	Lymphoblast Cell	$3x10^5$ ml	16-20	ECACC/ AstraZeneca

The types of cell line and source are stated. The seeding density (per ml or cm^2) and proliferation time (time taken to complete a single round of cell division) are also noted.

2.2 Cell Culture

2.2.1 MSC Isolation

MSC samples were previously isolated and cryopreserved by Dr Ruth Morse and Dr Craig Donaldson. The procedure of MSC isolation is briefly highlighted here.

BM samples were broken up with a scalpel and washed with DMEM/LG until remaining material (bone) looked white. The suspension was centrifuged and resuspended in DMEM/LG. Marrow aspirates were overlaid onto an equal volume of Lymphoprep™ (Axis-Shield, Dundee, UK; density 1.077 ± 0.001 g / ml) and centrifuged at 600 x g for 35 mins at room temperature to separate the mononuclear cells (MNCs) from neutrophils and red cells. The MNC layer was harvested and washed twice in DMEM/LG.

Isolated MNCs were then centrifuged and resuspended in DMEM/LG supplemented with 1% penicillin and streptomycin and seeded at a density of 1×10^7 per 25 cm^2 flask. Flasks were incubated at 37 °C in the presence of 5% CO_2. Non-adherent hematopoietic cells were removed by media exchange after 3-5 days. Mononuclear cells were then maintained by weekly demi-depletion of culture medium until 70% confluence was reached. Cells were then cultured as described in section 2.2.2.

2.2.2 MSC and HS5 standard culture conditions

Both the MSC and the HS5 stromal cell line are adherent cells that were seeded in DMEM/F12 containing 10% heat inactivated foetal bovine serum (FBS), 2 mM glutamine, 100 U / ml penicillin and 100 µg / ml streptomycin at 37 ˚C in the presence of 5% CO_2. Following thawing of cells (see 2.2.4) or trypsinisation of MSC/HS5 (see section 2.2.5) cells were resuspended in 1 ml of medium and a trypan blue cell count (section 2.3.2) was performed. MSC cells were then seeded at 1 x 10^6 per 75 cm^2 flask in 10 ml of complete DMEM/F12 culture medium. Cell culture medium was replaced with fresh medium once a week. HS5 cells were seeded at 1 x 10^6 per 25 cm^2 flask in 5 ml of complete DMEM/F12 culture medium. Cell culture medium was replaced

with fresh medium every 2-3 days and when both MSC and HS5 cells covered about 80% - 90% of the tissue culture flask, they were trypsinised and passaged.

2.2.3 Multiple Myeloma and TK6 cell lines standard culture conditions

Prior to establishment of co-culture conditions, U266B1 and RPMI 8266 cell lines were cultured in RPMI 1640 containing 10% heat inactivated FBS, 4 mM glutamine, 100 U / ml penicillin and 100 µg / ml streptomycin (complete RPMI culture medium). TK6 cells were also maintained in this medium and all cultures were maintained at 37 ˚C with 5% CO_2. Following thawing of each cell line (see section 2.2.4) cells were resuspended in 1 ml of medium and a cell count was performed (section 2.3.2). The cells were then seeded at 3 x 10^5 cells / ml in 10 mls of complete RPMI 1640 culture medium. Cell culture medium was replaced with fresh medium twice a week for MM cell lines and three times a week for TK6 cells with all cells passaged every 4 days.

2.2.4 Thawing of Cryopreserved Cells

Cryopreserved MSC samples and cell lines were rapidly thawed with agitation in a 37 ˚C water bath. Cells were washed by adding relevant thawing medium (culture medium supplemented with 20% heat inactivated FBS) dropwise up to a total of 10 ml per 1 ml cryovial over the duration of approximately 10 mins. MSC and HS5 were thawed using low glucose DMEM/F12, whereas MM and TK6 cell lines were thawed using RPMI 1640. This enabled the dimethyl sulphoxide (DMSO) to diffuse slowly out of the cells. Cells were then centrifuged for 10 mins at 350 x g (Hettich, Universal 320 Centrifuge). The supernatant was removed and discarded and this step was followed by a second wash in relevant thawing medium before the cells were seeded as described in sections 2.2.2 and 2.2.3.

2.2.5 Trypsinisation of the adherent MSC/HS5 cells

Trypsinogen (Trypsin), a proteolytic enzyme, was used to dissociate adherent cells from the flask. When the cells had reached 80-90% confluence (surface

of the flask almost covered by a cell monolayer) the medium was removed from the flask and the cells were washed once with phosphate buffered saline (PBS) (NaCL 8.0 g / l, KCL 0.2 g / l, disodium hydrogen phosphate 1.15 g/l, potassium dihydrogen phosphate 0.2 g / l). Trypsin (500 Nα-Benzoyl-L-arginine ethyl ester (BAEE) units porcine trypsin and 180 µg / ml EDTA) was then added to the flask at a volume sufficient to cover the surface of the cells. The flask was then gently agitated before incubation at 37 ˚C for 10 mins. After incubation the cells were observed to ensure detachment from the flask. The cells were then added to a 15 ml tube containing 10% FBS in DMEM/F12 to deactivate and wash off the trypsin and centrifuged at 350 x g for 10 mins. The cells were then suspended in complete culture medium and viable cells counted using trypan blue. Cells were then seeded at their relevant densities as mentioned in section 2.2.2.

2.2.6 Cryopreserving Cells

Cells were cryopreserved for future analysis by removal of all medium from the cell pellet and suspended in a freezing medium comprising 25% FBS and 10% DMSO in the relevant culture medium (DMEM/F12 or RPMI 1640). The DMSO was added dropwise over ice until cells were at a final concentration of 30 x 10^5 per ml for MM / TK6 cell lines and 1.5 – 2 x 10^6 for MSC and HS5. Cell suspensions were then added to cryovials at 1ml per vial. These were then placed in a cryopreserving chamber (‘Mr Frosty’) containing isopropanol which reduced the temperature gradually (1˚C per 1 minute) to -80 ˚C for 24 hrs before being transferred to vapour phase liquid nitrogen for storage.

2.2.1 Establishment of culture conditions

As MSC, HS5 and MM cell lines are known to have different preferred culture conditions and were to be cultured together in this study, a range of culture media and conditions were tested to enable optimal support of all cells in co-culture.

In order to establish a suitable co-culture medium, three types of complete medium were used for the MSC, with high glucose DMEM also assessed in

HS5 and MM cells. Proliferation rates (section 2.3.2) and morphologies (section 2.4) were compared.

This complete medium consisted of the following:

1. DMEM/F12 - Dulbecco's Modified Eagle's Medium supplemented with Nutrient F12 Ham, 10% heat inactivated FBS, 2 mM glutamine, 100 U / ml penicillin and 100 µg / ml streptomycin.

2. DMEM/LG - Low glucose Dulbecco's Modified Eagle's Medium, 10% heat inactivated FBS, 2 mM glutamine, 100 U / ml penicillin and 100 µg / ml streptomycin.

3. DMEM/HG - High glucose Dulbecco's Modified Eagle's Medium, 10% heat inactivated FBS, 2 mM glutamine, 100 U / ml penicillin and 100 µg / ml streptomycin.

4. RPMI 1640 - Roswell Park Memorial Institute Medium 1640, 10% heat inactivated FBS, 4 mM glutamine, 100 U / ml penicillin and 100 µg / ml streptomycin.

After assessing cell growth in each medium, DMEM/F12 complete medium was considered the most appropriate for this study (see chapter 3). The following methodology sections will refer to the culture conditions of all cells used following the optimisation conditions.

2.3 Chemotherapeutic agents

The effects of exposure of MSC, HS5 and U266B1 to several chemotherapeutic agents used in the treatment of MM were investigated using an *in vitro* treatment model. The concentrations used for each chemotherapeutic agent were determined from published data of plasma concentrations from patients undergoing intensive high-dose chemotherapy treatment or pre-stem cell transplant conditioning.

The following chemotherapeutic agents were tested using the clinically relevant dose: melphalan, 33 µM (Salehan, 2012), thalidomide, 200 ng / ml

(Kamikawa *et al*., 2006), lenalidomide, 4 μM (Chen *et al*., 2013), bortezomib, 500 nM (Moreau *et al*., 2011) and carfilzomib, 13.8 nM (Badros *et al*., 2013).

All drugs were stored at -80°C and thawed to room temperature before use.

2.3.1 Treatment conditions

All experiments, apart from the resistant cell line, have been performed with low passage cultures prior to the 20th sub-cultivation of the respective cells.

Initially MSC/HS5 were seeded in six wells of a 12 well plate (Corning, UK) at a density 2.0 x 10^4 cells per cm^2 for MSC and 4.0 x 10^4 cells per cm^2 for HS5. Cells were incubated for 24 hrs to enable adherence to the plate. U266B1 cells were then seeded at a density of 5 x 10^5 cells per ml of medium either alone or in a co-culture with the MSC/HS5 and were left for a further 24 hrs. Inserts (Millipore/Fisher, UK) with a pore size of 0.4 μm were added to two of the MSC/HS5 wells and 5 x 10^5 U266B1 cells were placed inside the insert in 1 ml (figure 2.1).

Treatment with each of the chemotherapeutic agents was given at the clinical dose and added for a period of 1 hr. After 1 hr the treatment was removed, cells were washed with PBS and 2.5 x 10^4 cells (calculated by trypan blue exclusion) were taken from each of the combinations (MSC/HS5 alone, U266B1 alone, MSC/HS5 from co-culture with U266B1 and U266B1 cells from co-culture with MSC/HS5) for comet analysis and 2.0 x 10^4 cells for micronucleus analysis.

The remaining cells were then administered with 1 ml of fresh DMEM/F12 medium and left to incubate for up to 72 hrs. A bystander model was also set up whereby U266B1 cells and MSC/HS5 previously exposed to drug alone, were cultured (separated by an insert) with a group of MSC/HS5 or U266B1 cells respectively that were not previously exposed. Cells were then sampled again for comet and micronucleus analysis at 16/24, 48 and 72 hrs following the same procedure performed at 1 hr.

Pre and during treatment

Type of Culture	Wells			
Independent	HS5 (Treated)	HS5 (Untreated)	U266B1 (Treated)	U266B1 (Untreated)
Co-culture	HS5/ U266B1 (Treated)	HS5/ U266B1 (Untreated)	U266B1 (Treated)	U266B1 (Untreated)
Co-culture – Bystander model	HS5 (Treated)	HS5 (Untreated)	BLANK WELL	BLANK WELL

Post treatment

Type of Culture	Wells			
Independent	HS5 (Treated)	HS5 (Untreated)	U266B1 (Treated)	U266B1 (Untreated)
Co-culture	HS5/ U266B1 (Treated)	HS5/ U266B1 (Untreated)	BLANK WELL	BLANK WELL
Co-culture – Bystander model	HS5 (Treated)/ U266B1 (Untreated)	HS5 (Untreated)/ U266B1 (Treated)	BLANK WELL	BLANK WELL

Figure 2.1 The experimental set up of a 12 well culture plate used in this study. To examine the interaction of MM cells with the BM microenvironment, three separate conditions were used, independent cultures, co-culturing MSC/HS5 with the MM cell line U266B1, and co-culturing the cells following exposure to chemotherapeutic agent to one cell compartment. MSC/HS5 were seeded into 6 wells of a 12 well plate and left to adhere for 24 hrs. U266B1 cells were then seeded into 4 wells independently and into 2 wells with MSC/HS5 separated by an insert and were left for 24 hrs. Cells were then either exposed to chemotherapeutic agent for 1 hr or left untreated. An untreated control was left adjacent to each treated sample. Cells were then washed free of drug and reseeded, two of the independently cultured U266B1 (treated and untreated) wells were then co-cultured via an insert with MSC or HS5 that had been treated or untreated independently previously. The treated U266B1 were cultured with the previously untreated HS5 and the untreated U266B1 were cultured with the previously treated HS5. Cells were then subsequently harvested for analysis immediately (1 hr), 16 or 24, 48 and 72 hrs post exposure to chemotherapeutic agent.

2.3.2 Trypan blue exclusion assay

This method is based on the principle that live cells (viable) possess intact cell membranes that exclude certain dyes, such as trypan blue, whereas in dead cells (non-viable) the cell membrane loses its integrity allowing the dye inside the cell (Tennant 1964, cited by Avelar-Freitas *et al*., 2014). A viable cell will have a clear cytoplasm whereas a non-viable cell will have a blue cytoplasm.

Prior to trypan blue staining, 10 µL of the well-mixed cell suspension was transferred to a sterile 1.5 mL tube. An equal volume of 0.4% trypan blue in 0.85% saline solution was added to the cell suspension and the mixture was incubated at room temperature for 1–2 mins. A small volume (10 µl) was then placed onto either a haemocytometer and counted under light microscopy or a cell counting slide (LUNA™, Labtech, UK) and counted using an automated cell counter (LUNA™, Labtech, UK). The haemocytometer is a thick glass microscope slide with an indented chamber of precise dimension that allows a defined volume of suspension to be deposited within the chamber where the cells can be counted under a general light microscope. A haemocytometer was used for general passaging of cells. The automated cell counter can record cell viability, concentration and cell count as well as cell size and was used during specific experiments.

2.4 Microscope examination

2.4.1 Light and phase contrast microscopy

Light and phase contrast microscopy were used to examine morphology and cell growth throughout the time course of each experiment. Images were taken using a Nikon Eclipse TE300 microscope and a Nikon Coolpix 950 camera.

2.4.2 Scanning electron microscopy

To examine the morphology of U266B1 cells and MSC when seeded on either side of an insert, scanning electron microscopy was used (section 3.3.6).

MSC were seeded at 2.0 x 10^4 cells / cm^2 on the underside of a 0.4 µm insert (Millipore/Fisher, UK) and left to incubate for 4 hrs at 37°C, in 5% CO_2. The

insert was then returned to its original position and placed in a 12 well plate with 1 ml of complete culture medium (section 3). U266B1 cells were then added to the 'basket' of the insert at a density of 5 x 10^5 cells / ml in 1 ml of culture medium.

Following 24 hrs, inserts were removed from the 12 well plate and residual medium within the insert was allowed to drain through the insert membrane (30-45 mins) before cells were washed in PBS (30-45 mins). Cells were then fixed in 4% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4 and left for 1 hr at room temperature. The fixative was then drained through the insert (30-45 mins) and rinsed in 0.1 M sodium cacodylate buffer three times each for 1 hr at 4°C. The sample was then stored in the fridge for 24 hrs in the final wash of buffer. The 0.1 M sodium cacodylate was drained through the insert. The samples were then dehydrated through a graded ethanol series (20%-100%) with a final wash in 100% ethanol three times.

The insert was then rinsed in hexadimethylsilazane (HMDS) (Acros Organics/Fisher, UK) and ethanol in the following ratios, 2:1, 1:1 and 1:2. The insert was then washed in 100% HMDS for a final three times and air dried overnight. Afterwards inserts were cut out and placed onto carbon conductive tabs (TAAB, UK) on pin stubs (TAAB, UK) and sputter coated with gold by a SC 500 Emscope sputter coating unit. Samples were observed using a Phillips XL30 environmental scanning electron microscope.

2.4.3 Confocal microscopy

Lipophilic tracers - long chain dialkylcarbocyanines DiI and DiO (Thermo Scientific) were used to visualise MSC and HS5 stromal cells that were seeded separately on the underside of cell culture inserts. MSC/HS5 were seeded onto the underside of a 0.4 µm insert membrane and were placed in the well of a 12 well plate and covered with 1 ml of DMEM/F12 medium. After 24 hrs, 5 µl of both DiI and DiO lipophilic tracers was added to the culture medium for 20 mins. The culture medium was removed and replaced with fresh medium three times. The insert membrane was then removed and placed on top of a microscope slide. A drop of immersion oil was added and a coverslip placed

on top. Images were taken at x40 magnification, using a PerkinElmer UltraVIEW, confocal microscope and UltraVIEW ERS software.

2.5 Genotoxicity Assays

2.5.1 *In vitro* micronucleus assay

The *in vitro* micronucleus assay is a genotoxicity test for the detection of small membrane bound DNA fragments i.e. micronuclei in the cytoplasm of interphase cells. These micronuclei may originate from acentric fragments (chromosome fragments lacking a centromere) or whole chromosomes which are unable to migrate with the rest of the chromosomes during the anaphase of cell division (Kirsch-Volders *et al*., 2000).

The Organisation for Economic Co-operation and Development (OECD) test guidelines allow the use of protocols with and without the actin polymerisation inhibitor cytochalasin-B. Cytochalasin-B allows for the identification and selective analysis of micronucleus frequency in cells that have completed one mitosis.

2.5.1.1 Cell preparation

At the end of chemotherapy treatment U266B1 cells were removed, and HS5 cells were washed with PBS and trypsinised (section 2.2.5). A volume of both cell types containing 2 x 10^4 cells was sampled and made up to 150 µl with PBS.

2.5.1.2 Slide preparation and staining

Initially microscope slides were soaked in 100% ethanol and polished. Slides were then labelled and placed inside a cytofunnel. The 150 µl sample of cells in PBS was then added to the cytofunnel (Thermo Scientific, UK). Slides were centrifuged at 103 x g in a cytospin (Thermo Scientific, UK) for 8 mins with acceleration set to high. After centrifugation the slides were removed and allowed to air dry. The slides were then fixed in 90% methanol for 10 mins. After thorough air drying the slides were either stained straightaway or stored at 37°C for future staining.

Phosphate buffer (0.66% w/v potassium phosphate monobasic and 0.32% w/v sodium phosphate dibasic, pH 6.4 – 6.5) was prepared fresh upon staining of slides. Slides were dipped in phosphate buffer before staining in acridine orange (12 mg / 100 ml in phosphate buffer) for 45 seconds. Staining was performed in a light proof coplin jar. Slides were then removed and washed in phosphate buffer for 10 mins followed by a further wash in fresh phosphate buffer for an additional 15 mins. Slides were allowed to air dry completely before analysis.

2.5.1.3 Slide Analysis

Immediately before scoring slides, a drop of fresh phosphate buffer was placed on top of the stained slides and a cover slip placed on top. Magnification was set to x40 in order to visualise cells. Slides were scored using a Nikon TE300 fluorescent microscope with a texas red (630/660 nm) emission filter. Images were captured using a Nikon Digital Sight DSFI 1 camera and NIS Elements software (Nikon Instruments Europe).

Two thousand mononuclear cells per replicate were examined and the number of micronucleated, bi-nucleated, multinucleated, necrotic, apoptotic and lobed nuclei cells per 2000 cells was recorded. Figure 2.2 outlines the typical morphologies seen.

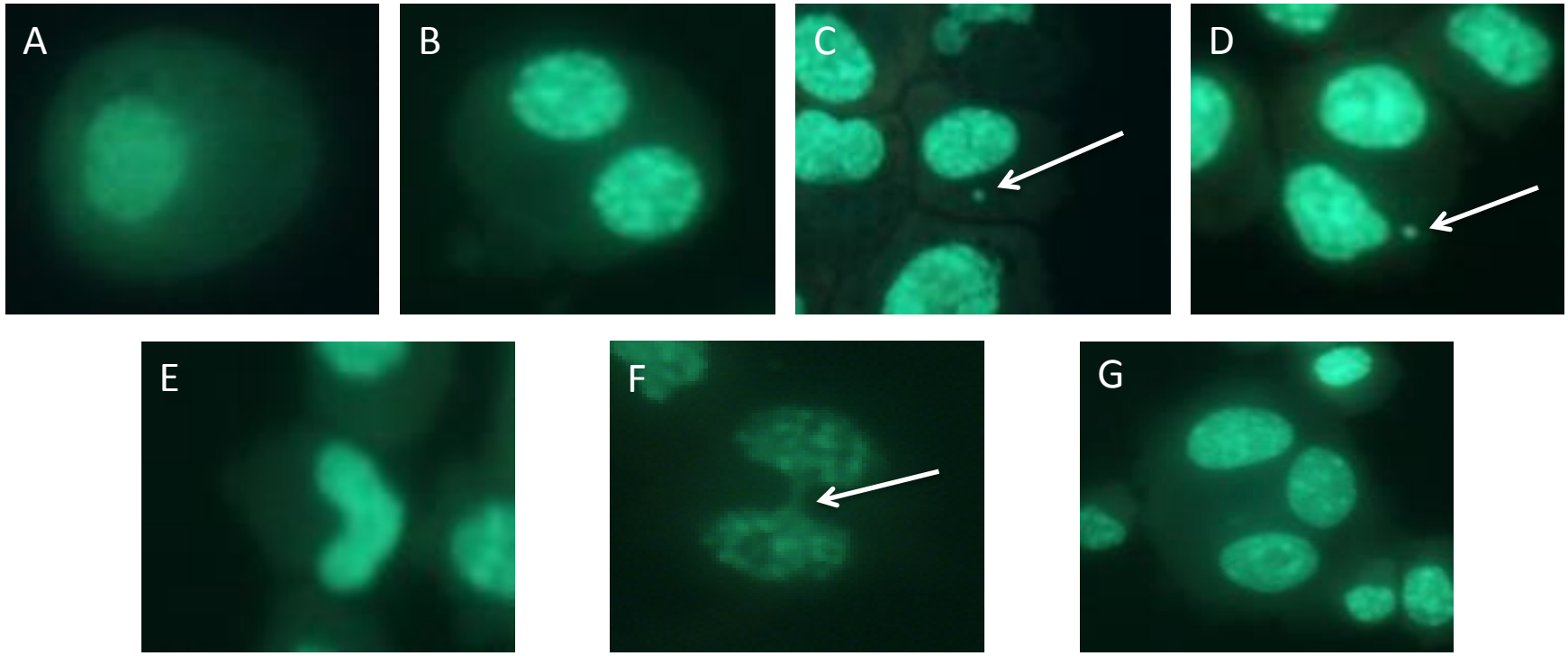

Figure 2.2 Photomicrographs of the typical morphologies seen when scoring MN slides. Nuclear material fluoresces green and cytoplasm fluoresces orange/light green. Normal mononuclear cells (A), bi-nucleated cell (B), mononuclear cell with micronucleus (arrowed) (C), bi-nucleated cell with micronucleus (arrowed) (D), lobed nuclei (E), cytoplasmic bridge (F), multinucleated cell (G). All images X 40 magnification.

2.5.1.4 Scoring of micronuclei

The following criteria for scoring micronuclei were adopted from Fenech (1996):

1. Diameter less than one third of main nucleus,

2. Micronuclei should be separate from or marginally overlap with main nucleus as long as there is clear identification of the nuclear boundary, and

3. Micronuclei should have similar staining as the main nucleus

As cytochlasin – B was not used cytotoxicity evaluated based on Relative Increase in Cell Counts (RICC) as recommended by OECD guidelines. RICC was calculated using the following equation:

$$RICC = \frac{\text{(Increase in number of cells in treated cultures (final – starting))}}{\text{(Increase in number of cells in control cultures (final – starting))}} \times 100$$

2.5.2 Comet assay

In order to assess for DNA damage in the cell lines and the MSCs following chemotherapeutic treatment, the single-cell gel electrophoresis (comet assay) was used.

McNamee *et al*. (2000) were the first to describe how comet assay agarose gels could be attached to a coated polyester film, thus replacing the glass slide. The Gelbond® film (Lonza, UK) is a thin stable surface used as a support for agarose gels in general and provides a suitable, more effective surface for comet assay agarose gels. As low melt agarose adheres directly to its surface, the need to pre-coat the casting surface is eliminated and thus decreases the unexpected loss of samples (as often occurs with glass slides).

2.5.2.1 Harvesting cells for comet analysis

At the end of chemotherapy treatment MM cells were removed from the plate/insert and centrifuged, adherent cells were washed with PBS and trypsinised. The trypsin was deactivated by the addition of culture medium. The cells were then centrifuged at 500 x g for 5 mins and suspended in 1 ml

of complete culture medium. An aliquot of each cell suspension (10 µl) was used for trypan blue assessment. Low melt agarose (0.5%, LMA) was melted in PBS and kept in a 37 °C water bath. Cells (2.5 x 10^5 cells) were combined with 180 µl liquefied LMA (37 °C), gently mixed by pipetting and 40 µl was immediately transferred onto Gelbond® film. A 22 x 22 mm glass cover-slip was gently placed onto each gel, to flatten out the molten agarose layer (two gels per sample). After solidifying at 4°C for 10 mins, the cover slips were removed and the gels placed in a pre-cooled lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris buffer (pH 10), 10% DMSO, 1% Triton X-100) in a light proof box overnight. This lysis solution removes cell membranes and cytosol, leaving the histone depleted DNA intact.

2.5.2.2 Electrophoresis

After lysis, the Gelbond® was removed from the lysis solution, drained and placed in a horizontal light proof gel electrophoresis tank (Fisher Scientific, UK) side by side, avoiding space. The tank was filled with fresh pre-cooled alkaline electrophoresis buffer (200 mM NaOH, 1 mM EDTA, pH> 13) covering the gels. Before electrophoresis the Gelbond® films were left for 20 mins to allow the DNA to unwind and produce single stranded DNA. After alkaline unwinding, the gels were electrophoresed under the same buffer conditions. The electrophoresis conditions were 0.7 V/cm, 300 mA for 20 mins. The electric current was maintained at 300 mA by adjusting the volume of electrophoresis buffer in the tank. The electrophoresis tank was kept at 4 ˚C using a recirculating water bath (Grant Instruments, UK). After electrophoresis the gels were immersed three times for 5 mins in neutralisation buffer (0.4 M tris-HCL (hydroxymethyl aminomethane hydrochloric acid), pH 7.5). The gels were then refrigerated in the third wash of neutralisation buffer in a light proof box prior to analysis.

2.5.2.3 Comet Evaluation/Analysis

Following electrophoresis, Gelbond® films were cut and placed onto microscope slides (Academy Science, UK) and each gel was then stained in the dark using 50 µl propidium iodide (20 µg / ml). Gelbond® film mounted

slides were visualised using a Nikon TE300 inverted fluorescent microscope fitted with a texas red filter and barrier filter. Magnification was set to x40 in order to visualise comets. Images were taken using a Nikon Digital Sight DSFI 1 camera and NIS Elements software (Nikon Instruments Europe). Slides were scored in a regular formation to ensure that no cell was scored twice. Fifty comets were scored per gel before September 2014 after which time one hundred and fifty comets were scored as per updated OECD guidelines (http:/www.oecd.org). Care was taken to avoid overlapping or potentially overlapping comets. Images were then analysed using Comet Assay IV software (Perceptive Instruments). Percentage of DNA in the tail was used to evaluate each comet.

2.5.2.4 Positive quality control; hydrogen peroxide (H_2O_2)

A second sample for each treated and untreated well was also set onto Gelbond® film before being immersed in 50 µM H_2O_2 for 5 mins in a light proof box on ice, prior to incubation in lysis buffer. H_2O_2 is commonly used as a positive control in the comet assay as it is capable of inducing DNA strand breaks and can thus confirm the capability of the assay in detecting DNA damage. The rest of the comet assay was performed as previously described in section 2.5.2.2 and 2.5.2.3.

2.6 Enzyme Linked Immunosorbent Assay (ELISA)

In order to measure levels of IL-6 released from the MSC, HS5 and U266B1 cells following co-culture and exposure to chemotherapeutics, an in-house sandwich ELISA was developed during this study.

Firstly each well of a 96 well immunosorbent plate (Nunc, Thermo Scientific, UK) was coated with 50 µl of IL-6 capture antibody (Ab) (3.5 µg / ml) and left to incubate at 4 ˚C for 24 hrs. Antibody details are listed in table 2.2. Plates were then washed with PBS / 0.1% tween 20 before each well was blocked by adding 200 µl PBS / 1% bovine serum albumin (BSA) per well and left to incubate for 1 hr at 37 ˚C. The blocking agent was then removed and the plates washed with PBS / 0.1% tween 20 followed by PBS. Next, 50 µl of standards were added to each well in duplicate. These comprised; a standard curve in

duplicate produced by double diluting recombinant cytokine (8 ng / ml (BD Pharmingen, UK)); a high quality control (750 pg / ml); a low quality control (75 pg / ml) (positive controls), and complete DMEM/F12 culture medium or PBS (negative controls). The remainder of the plate was filled with test samples in quadruplicate.

The samples were then incubated for 2 hrs at room temperature. After washing with PBS / 0.1% tween 20, the biotinylated rat anti-human detection Ab (BD Pharmingen) was added at 1.75 µg / ml and left to bind for 1 hr at room temperature. Again the plate was washed (PBS / 0.1% tween 20) before 50 µl of Pierce streptavidin PolyHRP (0.5 mg / ml) was added to each well. The plate was then left to incubate for 30 mins at room temperature before 100 µl of substrate (0.1 mg / ml tetramethylbenzidine (TMB) in 0.1 M citrate-phosphate buffer, containing 0.03% hydrogen peroxide) was added. When the blue colour started to show in the penultimate well, 50 µl of 2 M sulphuric acid was added to stop the reaction. The ELISA was then analysed using a Fluostar Optima spectrophotometer (BMG Labtech, UK) at 450 nm, with 595 nm as a reference to remove interference caused by the immunoplate.

Table 2.2 – Details of antibodies used for ELISA in this study.

Cytokine	Purified Ab (Isotope, Clone, Concentration (µg/ml))	Biotinylated Ab (Isotope, Clone, Concentration (mg/ml))	Recombinant Cytokine (ng/ml)	Manufacturer
IL-6	Mouse IgG1, MQ2-13A5, 3.5 µg / ml	Rat IgG2a, MQ2-39C3, 1.75 mg / ml	8	BD Pharmingen, UK

The types of each antibody used in this study and source are stated.

2.7 Functionality Assays

2.7.1 Cell Differentiation

MSCs are important cellular components of the BM microenvironment and under appropriate culture conditions *in vitro* can be stimulated to differentiate into a variety of mesenchymal tissues. HS5 stromal cells were cultured to differentiate into either adipogenic or osteogenic mesenchymal lineages *in vitro* by appropriate media in a 37 °C, 5% CO_2 incubator according to the manufacturer's instructions.

For both adipogenic and osteogenic differentiation HS5 cells were seeded alone or in co-culture with U266B1 cells. On day one HS5 cells that were to be stimulated along an adipogenic lineage were seeded at 5 x 10^4 cells per cm^2 in DMEM/F12 in a 12 well plate (Corning, UK) for 24 hrs. HS5 cells that were to be stimulated along an osteogenic lineage were seeded at 3 x 10^4 cells per cm^2 in DMEM/F12 also in a 12 well cell culture plate (Corning, UK) for 24 hrs.

After 24 hrs for both adipogenic and osteogenic cultures, U266B1 cells were seeded into inserts at 5 x 10^5 cells per ml in 1 ml of medium, inside these wells and left to incubate for 24 hrs. Both HS5 alone and co-cultures were exposed to a clinical dose of chemotherapeutic agent for 1 hr (section 2.3). Cells were washed free of drug and HS5 to be stimulated into adipocytes were maintained in AdipoDiff medium (StemMACS, UK) with HS5 to be stimulated into osteocytes maintained in OsteoDiff medium (StemMACS, UK). Both adipogenic and osteogenic cultures were maintained by complete medium changes every 3-4 days. U266B1 cells that were co-cultured with HS5 remained in co-culture for the duration of the assay.

On day 10, HS5 cells stimulated to differentiate along an osteogenic lineage were stained for the presence of alkaline phosphatase (ALP) with SIGMA FAST BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium) substrate which forms an insoluble blue precipitate in the presence of ALP. U266B1 cells that were co-cultured with HS5 cells were removed and HS5 cells from the co-culture and independent cultures were washed twice with

PBS to remove residual medium and incubated with pre-cooled methanol for 5 mins. HS5 were then washed in de-ionized water and incubated with SIGMA FAST substrate for 10 mins on a plate shaker at room temperature, before a final wash in de-ionized water.

On day 21, HS5 cells stimulated to differentiate along an adipogenic lineage were assessed. U266B1 cells that were co-cultured with HS5 cells were removed and HS5 cells from the co-culture and independent cultures were washed twice with PBS to remove residual medium and incubated with pre-cooled methanol for 5 mins. HS5 were then washed with de-ionized water. They were then stained for lipid containing vacuoles with 0.3% w/v Oil Red O (1-([4-(Xylylazo)xylyl]azo)-2-naphthol,1-[2,5-Dimethyl-4 (2,5dimethylphenylazo)phenylazo]-2-naphthol, Solvent Red 27) in isopropanol for 20 mins on a plate shaker at room temperature, washed again in de-ionized water and examined immediately.

All cultures were examined immediately after staining using a Nikon Eclipse TE300 microscope and colour images were taken on a Nikon Coolpix 950 camera.

2.7.2 Flow cytometry

HS5 cells were cultured at 1 x 10^6 cells per 25 cm^2 flask, treated with chemotherapeutic agents and evaluated for the cell-surface expression of CD45, CD73, CD105, CD34 and CD14. Control flasks without chemotherapeutic agent were used to assess normal expression levels. HS5 were exposed to chemotherapeutic agents for one hr, then washed with PBS, trypsinised (section 2.2.5) and reseeded in DMEM/F12 complete medium. Cells were sampled for flow cytometry at 72 hrs post exposure.

Cells (1.5 x 10^5) were washed in PBS and centrifuged at 500 x g for 5 mins to remove any residual medium before being suspended in 100 µl MACS buffer (0.5% BSA and 0.04% EDTA, pH 7.2) and stained with 5 µl of each Ab or isotype control for 45 mins at 4 °C. Subsequently excess antibody was washed off with PBS before cells were fixed in 1% paraformaldehyde / 1% BSA in MACS buffer. Cells were analysed by flow cytometry (BD Accuri™ C6

cytometer; Becton Dickinson, Oxford, UK). Cells were gated to exclude debris and analysis was based on collecting 10,000 events through the gate. HS5 cells were gated based on forward scatter and side scatter properties. FITC labelled cell signals were resolved on channel FL1. PE labelled signals were resolved on channel FL2.

Each of the following antibodies were conjugated with Phycoerythrin (PE): anti-CD105 (AbD Serotec, UK), anti-CD73 (BD Biosciences, USA), anti-CD14 (BD Biosciences, USA), or Fluorescein isothiocyanate (FITC): anti-CD45 (BD Biosciences, USA) and anti-CD34 (AbD Serotec, UK) as well as isotype controls (all BD Biosciences). All antibodies were mouse antihuman IgG1 κ except anti CD14 which was IgGIb κ.

2.8 Mitotic Analysis

2.8.1 Thymidine Double Block for Cell Synchronisation

In order to synchronise TK6 cells for cell cycle analysis, a thymidine double block was performed on these cells. TK6 cells were seeded at a density of 3 x 10^5 per ml in a 75 cm^2 flask in complete DMEM/F12. Thymidine (100 mM) was then added to the culture and left to incubate for 24 hrs. This was the first block. The culture medium was then removed and the cells were placed in fresh culture medium for 8-10 hrs after which time a second block of thymidine (100 mM) was added and left to incubate for 15-16 hrs. TK6 cells were then washed with 2 ml of complete RPMI 1640 medium 3 times before cells were exposed to thalidomide (200 ng / ml) or lenalidomide (4 µM) for 1 hr. Cells were then washed with fresh culture medium and reseeded, with a sample of 2 x 10^4 cells removed for analysis by flow cytometry every hour for 6 hrs.

2.8.2 Propidium iodide staining for cell cycle analysis

TK6 cells were harvested, washed in PBS and centrifuged at 380 x g for 5 mins. The supernatant was removed and the pellet was resuspended in 70% ice cold ethanol and left overnight at 4 °C to fix. Upon analysis cells were washed with PBS and suspended in propidium iodide (PI) staining buffer (containing 10 µg / ml RNAse A and 50 µg / ml PI in PBS). Each sample was

left to incubate at room temperature in the dark for 30 mins. NaCl (0.13 mM) was added to stop the reaction. Cells were analysed on a BD Accuri™ C6 flow cytometer.

2.9 Statistical Analysis

All values are represented as mean ± standard deviation of the mean. All values are representative of 3 biological repeats unless otherwise stated. All statistical analysis other than that of ELISA have been calculated using raw data in Microsoft Office Excel software. ELISA statistical analysis and graphical representation was done using GraphPad Prism 6.0 software. All samples were confirmed to be normally distributed using the Shapiro-Wilk test. An unpaired Student's t-test was used to compare samples against a relevant control at each time point. When comparing samples between time points a two way ANOVA was used determine whether there was a significant difference between the associated means. A p-value of 0.05 or below was considered significant. Graphical presentation of significant differences was identified as (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.