Characterisation of Extracellular Vesicles from the Microenvironment in High Risk Acute Myeloid Leukaemia

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Abstract

Haematopoietic stem cell transplantation is the most effective anti-leukaemic therapy for AML for a large number of patients, but a significant proportion of these will relapse post-transplant with a poor prognosis for long-term survival.

The bone marrow microenvironment has been implicated as a major contributor to chemotherapy resistance and relapse through mediating communications between residual cells which have been shown to preferentially support and maintain the leukaemic niche.

Interactions within this malignant niche can be facilitated by exosomes, extracellular vesicles secreted by multiple cell types that function as delivery vehicles for mRNA, DNA, miRNA, enzymes and cytokines. The ability of secreted exosomes to induce microenvironmental changes that may differentially support normal or malignant stem cells in the post-transplant setting is relatively unknown.

Characterisation of exosomes originating from MSCs revealed exosome particle number and protein content was significantly increased in diagnostic MSC samples compared to normal and post-BMT samples, along with miRNA yield which was also found to be significantly higher in this patient sub-set.

Ex vivo co-culture assays using functional exosome preparations from primary AML-MSCs revealed several phenotypic effects including exosome induced proliferation when co-cultured with primary AML blasts, cell adhesion and a significant protection against drug treatment.

Secreted cytokine profiling by Luminex bead capture array showed that exosome cytokine profile change from adhesion related within the NBM and diagnostic samples, to immunology related targets in early-BMT and adhesion and survival/differentiation related in late-BMT. This change reflects a stabilization of the inflammatory environment towards NBM levels, along with an increase in adhesion related molecules suggesting a recovery post-transplant and potential early indication of disease relapse, GvHD or GvL.

These results demonstrate how important extracellular vesicles are in creating a hostile microenvironment as promotors of residual disease, and for the first time the malignant potential of microenvironment derived MSC exosomes in AML.

Acknowledgements

List of Abbreviations

AML	acute myeloid leukaemia
AKT	protein kinase B (PBK)
ANOVA	Analysis of variance
APC	allophycocyanin
ATP	adenosine triphosphate
ALIX	Apoptosis-linked gene 2-interacting Protein X
BCA	Bicinchoninic acid
BM	bone marrow
BSA	bovine serum albumin
CAR	CXCL12-abundant reticular
CBF	core binding factor
CEBPA	CCAAT/enhancer binding protein (C/EBP) alpha
CML	chronic myeloid leukaemia
CR	complete remission
D_2O	Deuterium oxide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNMT	DNA (cytosine-5-)-methyltransferase 3 alpha
EC_{50}	50% inhibitory concentration
EDTA	ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ESCRT	Endosomal sorting complex required for transport
EV	Extracellular vesicle

ERK	extracellular regulated kinase
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FLT3	FMS-like tyrosine kinase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCSF	granulocyte colony stimulating factor
HER2	Human epidermal growth factor receptor 2
HSC	haematopoietic stem cell
IL	interleukin
IMDM	Isocove's modified Dulbecco's medium
ITD	internal tandem duplication
LSC	leukaemic stem cell
LDS	lithium dodecyl sulphate
mRNA	Messenger ribonucleic acid
MAPK	mitogen-associated protein kinase
MEM	minimal essential medium
MM	Multiple Myeloma
miRNA	microribonucleic acid
MRD	minimal residual disease
MSC	Mesenchymal stem cell
NTA	Nanoparticle Tracking Analysis
HS-5	human stroma-5
mTOR	mammalian target of rapamycin
NPM-1	nucleophosphim-1
OS	overall survival
PB	peripheral blood

PBS	phosphate buffered serum
PDGFR	platelet derived growth factor
PDK-1	3-phosphoinositide-dependent protein kinase 1
PE	phycoerythrin
PI	propidium iodide
PI3K	phosphatidyl-inositol 3'-kinase
PKC	protein kinase C
PR	partial remission
PVDF	polyvinylidene difluoride
RAS	reticular activating system
RIPA	Radioimmunoprecipitation assay
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SEM	standard error of the mean
STAT	signal transducer and activator of transcription
TBS	tris-buffered saline
TIMP	Tissue inhibitor of metalloproteinase
TKD	tyrosine kinase domain
TRF	Time resolved fluorescence
VEGF	vascular endothelial growth factor
W/V	Weight per volume

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1 Chapter 1: Introduction

1.1 The bone marrow microenvironment

The bone marrow microenvironment or niche is a complex network of heterogeneous cell populations that function either to support or are directly involved in haematopoiesis. The niche is home to haematopoietic stem cells (HSCs) which reside here either in long-term quiescence or in a transient self-renewing state until triggered by cell death or damage at which point the HSCs withdraw from a quiescent state and actively start dividing again, this transition is regulated by several signalling pathways including Wnt and the MEK/ERK pathway (Baumgartner et al. 2018). HSCs give rise to common intermediate lymphoid or myeloid progenitor cells, which then further differentiate into lymphocytes, megakaryocytes/erythrocytes or granulocyte/macrophages which are then released into circulation for a fully functioning haematopoietic system (Figure.1.1).
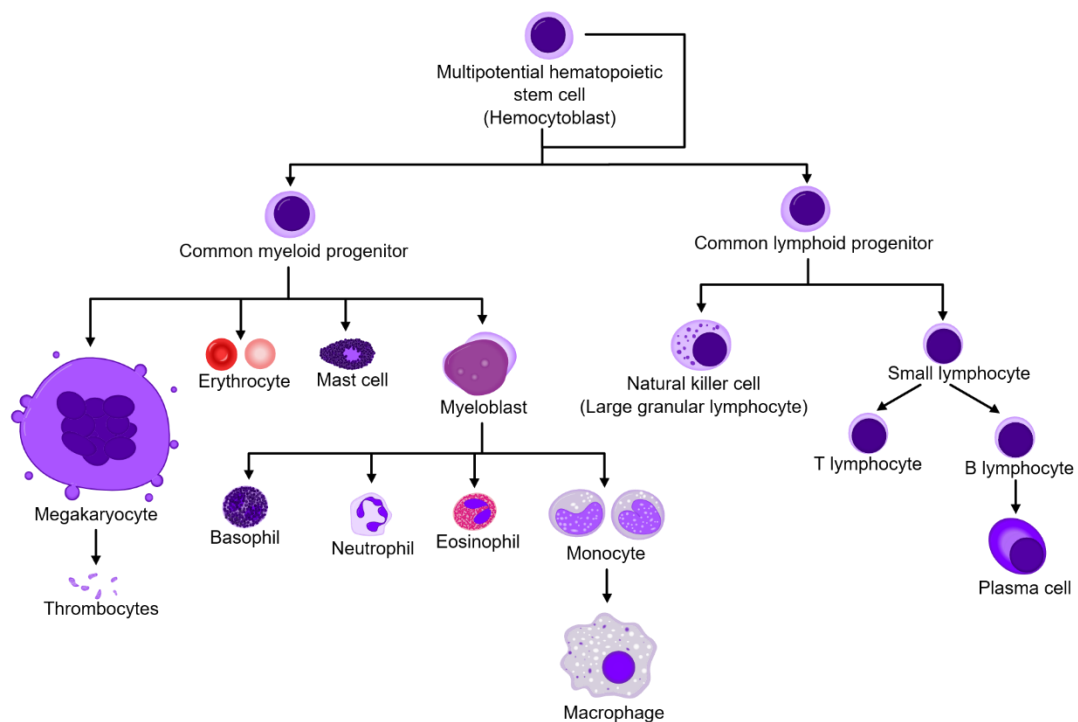
Figure 1. 1 Schematic representation of haematopoiesis (Haggstrom.M et al, 2009)

HSCs are supported on a meshwork of stromal tissue which lines the bone marrow microenvironment and consists primarily of multipotent mesenchymal stem cells (MSCs) which are responsible for establishing this haematopoietic region (Mendez-Ferrer et al. 2010). MSCs have the capacity to differentiate into many supportive call

types – adipocytes (primarily function as fat storage vehicles but also operate as a negative regulator of haematopoiesis), osteoblasts (synthesise bone tissue and regulate bone marrow angiogenesis) and chondrocytes (synthesise cartilaginous tissue). Two other important supportive cell types are endothelial cells (enable exchange of molecules between blood and bone marrow) and fibroblasts (synthesise structural components of bone marrow).

The niche can be separated into two distinct parts; the endosteal and vascular niche, both regulate HSCs but in different ways (Perry and Li 2007). The endosteal niche is made up of osteoblastic lineage cells and regulatory T-cells (Treg) which are a specialized sub-category of T-cells that function to prevent autoimmunity and promote homeostasis. Also present are sympathetic neurons which regulate HSC activity within the niche (Hanoun et al. 2015) as well as MSCs. Typically hypoxic with low blood perfusion, quiescent HSCs prefer this environment (Semenza 2007). Hypoxic inducible factors (HIF) are present on the cell surface of both HSCs and MSCs helping to maintain a quiescent state by activating numerous downstream target genes such as *Vegf* and *Cxcr4* (Andrade et al. 2015). The vascular niche is normoxic and consists primarily of MSCs and CAR (CXCL12-abundant reticular) cells which support the actively cycling HSCs which reside here (Winkler et al. 2010a).
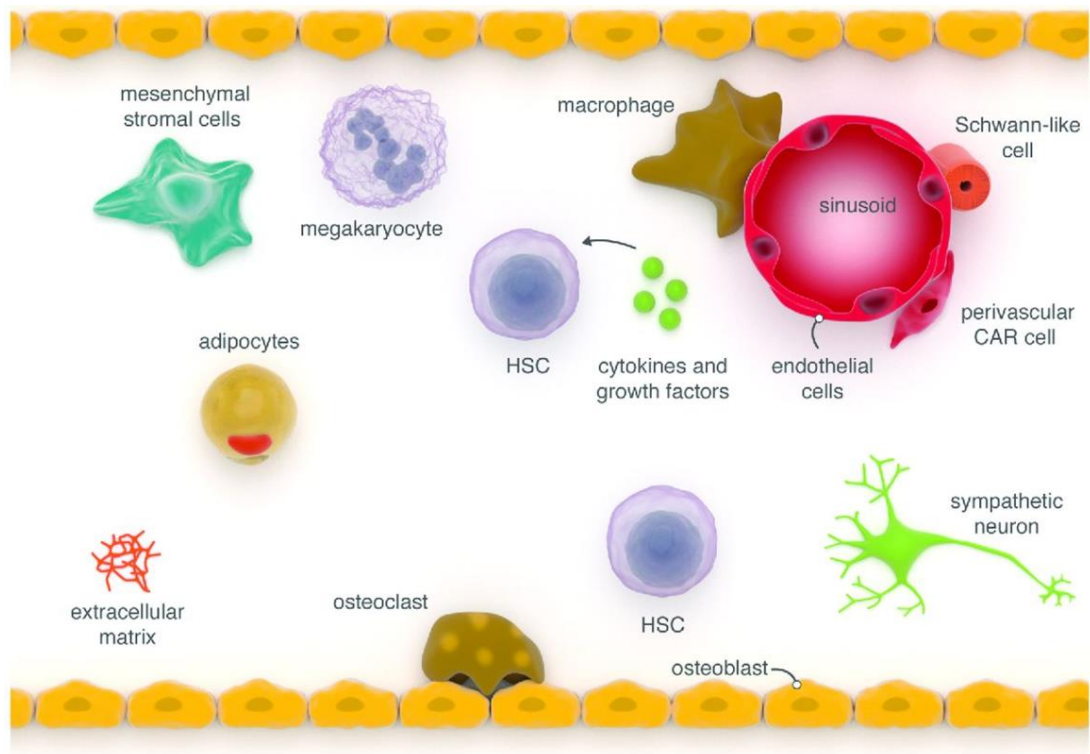
Figure 1. 2 Overview of cell types within the normal bone marrow niche (Krause and Scadden 2015)

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The supportive stromal cells help regulate normal haematopoiesis with the help of secreted soluble factors, they produce ligands and molecules that interact with their counterparts on the HSC surface which bring about several cellular functions. For example, CXCR4 is found on the surface of HSCs, is activated by the SDF-1 ligand which interacts with CXCL12 secreted by MSCs which brings about a chemotactic response. Increasing calcium levels then lead to a production of VLA-4 and LFA-1 that adhere to HSCs and increase cell adhesion and retention (Kondo et al. 2003; Drury et al. 2011; Ostanin et al. 2011).

1.2 Microenvironment and Cancer

There are many ways the bone marrow microenvironment (BM-ME) contributes to cancer progression, it's remodelling by invading cancer cells can provide a sanctuary site to enhance cancer cell proliferation and protect from the immune system. Within the BM-ME MSCs are known to function as a promotor of cancer metastasis and tumours recruit inflammatory cells via the expression of chemokines resulting in increased tumour survival, differentiation and vascularization of the tumour microenvironment (Joyce and Pollard 2009). The BM-ME has also been identified as a site of protection for cancer stem cells to reside, preserving their phenotypic plasticity, protecting from immunosurveillance and facilitating metastasis (Plaks et al. 2015).

The bone marrow is the major site of haematopoiesis and bone formation and it plays a key role in perpetuating many haematopoietic malignancies. Leukaemic cells interact with cells of the bone marrow to create a microenvironment that is favourable by facilitating interactions through paracrine and autocrine signalling molecules to increase cell proliferation, survival, adhesion and consequently chemotherapy resistance and the potential onset of minimal residual disease (Garrido et al. 2001; Huntly and Gilliland 2005).

Alterations in the BM-ME have been reported in most human malignancies including breast, gastric and tumorigenesis and other haematological malignancies (Manier et al. 2012; Kawano et al. 2015; Purroy et al. 2015). Within tumorigenesis the BM-ME release of factors such as VEGFR-1 and VEGFR-2 act as a source of inflammation within the primary tumour, helping the formation of a pre-metastatic disease. Bone marrow derived VEGFR-1 positive cells alongside macrophages infiltrate hypoxic tumour regions and can induce CXCR-4 expression in endothelial cells of the BM-ME helping to promote tumour migration and angiogenesis (Reddy BY 2012). Gastric

cancer cells are supported within a stromal and extracellular matrix of the BM-ME providing adherent and secretory signals which help cancer stem cells initiate tumour growth (Ishimoto et al. 2014). The BM-ME is thought to play a major role in the common relapse that can take place within breast cancer years after remission. It is thought that dormant cancer cells exist within the BM-ME forming gap junction intercellular communication with stromal cells creating a preferential niche in which cancer cells can stay long term in a quiescent state avoiding therapeutic agents which target proliferating cells (Chantrain CF 2008). Tumour-associated macrophages (TAMs) within the BM-ME are known to be involved within a paracrine loop between cancer cells and endothelium cells forming clusters which lead to enhancement and dissemination of cancer cells, increased density of these clusters has seen to correlate with increased development of disease within breast cancer (Joyce and Pollard 2009).

 Within haematopoietic malignancies there are thought to be two possible mechanisms by which alterations take place; somatic mutations within the stromal cells or malignant haematopoietic cells generating signals that cause altered function (Yao and Link 2017). There is data to support both mechanisms, but it is thought to be more likely that the malignant haematopoietic cells target stromal cells through the expression of specific factors which induce alterations in the microenvironment and allow the selective expansion of diseased haematopoietic progenitor cells. This gives the leukaemic stem cells (LSCs) a competitive advantage over normal cells and these changes can influence stromal response to chemotherapy and create therapeutic resistance (Zhang et al. 2012).

1.3 Acute Myeloid Leukaemia

AML is the most common adult form of leukaemia and over the last decade it's incidence rates have increased by 8% in the UK to over 3000 new cases each year (Cancer Research UK, 2016).

AML is characterised by an accumulation of immature and poorly differentiated cells in the bone marrow and peripheral blood, this leads to a disruption of normal haematopoiesis and bone marrow failure (Gutierrez and Kentsis 2018). Malignant cells can also infiltrate other tissues including the lungs, CNS and soft tissue although this occurs less frequently.

AML can occur across a range of age groups although is predominantly associated with those over the age of 60. The mutation of a somatic stem cell subsequently develops into a leukaemic clone (Dash and Gilliland 2001). In addition to genetic

mutations, cytogenetic abnormalities can be detected in approximately 50-60% of newly diagnosed AML patients and are associated with certain prognostic groups. These usually consist of favourable (e.g. presence of inv16), intermediate (e.g. normal karyotype) and adverse (e.g. presence of monosomy 7) based on recognised risk-scores (Estey 2018). Those patients designated to adverse prognostic group require a more intensive therapeutic regime and are most likely to undergo allogeneic stem cell transplant.

The number of coding mutations per AML genome is on average 13 per person and are found to reoccur in many commonly deregulated pathways, 96% of AML patients have at least one driver mutation in one of these genes contributing to why AML is such a heterogeneous disease (Bullinger et al. 2017). Cytogenetic rearrangements are also common and are used to diagnose AML prognosis and tracking of the disease at a minimal residual disease level (MRD). (Papaemmanuil et al. 2016).

The abnormalities underlying AML are heterogeneous and new mutations commonly arise in response to treatment and at relapse, making it a very complex disease which is difficult to treat.

1.4 Treatment of AML

Treatment for AML patients is mainly with chemotherapeutic drugs that inhibit cell proliferation. Treatment usually consists of two phases, intensive induction therapy which aims to eliminate the majority of leukaemic cells and consolidation therapy which aims to maintain long term remission (Rowe 2009).

Older AML patients defined as over 65 years of age are much less likely to tolerate standard intensive cytotoxic treatment and over the last 50 years have shown limited improvement in treatment outcome with low dose regimes compared to the younger age patient group (Burnett 2012).

Due to the heterogeneous nature of AML along with other important prognostic factors such as cytogenetics and age, patient stratification is complex and essential in order to tailor treatment to achieve adequate therapeutic effect. For patients of adverse prognostic group especially there is an unmet clinical need for more effective therapies, especially since treatment has not changed significantly in the last 30 years. Bone marrow transplant may be the only option for poor prognosis patients, but for many elderly patients this is not viable (Duarte et al. 2018).

Diagnostic molecular information is used to track disease progression this is particularly important when monitoring MRD (minimal residual disease) and when

deciding on therapies which target specific molecular mutations for example isocitrate dehydrogenase (IDH) inhibitors used to treat IDH mutation (Koreth et al. 2009; Stone et al. 2017).

Standard treatment for AML consists of two phases, a remission induction phase and a consolidation therapy phase. The induction phase consists of a combination of cytarabine (Ara-C) treatment alongside an anthracycline, usually daunorubicin. Cytarabine is a pyrimidine analogue which functions by disrupting DNA synthesis resulting in a reduction of cell proliferation. Anthracyclines are antibiotics which function to reduce luekaemic burden (Dohner et al. 2010).

Following induction phase, if complete remission (CR) is reached patients will go on to receive post-remission therapy which consists of either consolidation chemotherapy or haematopoietic stem cell transplantation, or a combination of both. Treatment is based on the disease and patient characteristics and availability of a donor for transplant (Thol et al. 2015).

Advances have been made in the molecular characterization of AML subtypes allowing patients to receive more effective treatment, but the greatest challenge remains the effective targeting of relapsed disease. The standard therapy as outlined above incur significant toxicities and risk of disease recurrence is still high, new treatments which are lower in toxicity are particularly required for the elderly and those that are transplant ineligible (Altman and Platanias 2013).

Due to the challenges in treatment of AML studies have concentrated on developing therapies which identify specific targets which could potentially be used in combination with chemotherapy, a successful example of this is the targeting of FLT3, clinical trials with targeted FLT3 inhibitors report around a 50% response rate in patients harbouring a FLT3-ITD mutation (Wander et al. 2014). Other possible targets include; CREB a transcription factor which regulates gene expression and functions as a protooncogene in haematopoiesis, Triad1 is an E3 ubiquitin ligase and a target of HoxA10 know to have pro-leukaemogenic effects, Bcl-2 family critical regulators of the apoptotic pathway with a high association with AML, JAK/STAT pathway is commonly activated in myeloproliferative disorders which commonly progress to AML (Andreeff et al. 1999; Kralovics et al. 2005; Marteijn et al. 2005; Shankar et al. 2005).

1.5   Bone marrow transplantation in AML

Allogeneic stem cell transplantation is still the most effective anti-leukaemic therapy for adverse prognostic AML and over a third of haematopoietic stem cell transplants

(HSCTs) are performed as therapy for AML worldwide (Niederwieser et al. 2016). A significant proportion of patients will relapse post-transplant and the probability of long term survival following early relapse is less than 20% (Tsirigotis et al. 2016).

Assessment of patients for HSCT is based on several factors which include disease risk, patient comorbidity and the willingness of the patient to take part in the procedure. A prognostic scoring system is commonly employed to help aid decision making e.g. Haematopoietic Cell Transplant Co-morbidity Index (Christopeit et al. 2013).

Patients over 60 and adverse risk disease at high risk of relapse (70-90%) can be offered HSCT if generally fit with the introduction reduced intensity conditioning regimes (RIC). Standard approach when considering a HSCT for AML is to find a human leukocyte antigen (HLA) matched sibling donor, if this is not possible then an alternative donor may be considered.

AML transplant patients are stratified depending on their molecular subtype, this is important as it allows risk estimation and post-remission treatment to be identified appropriately (Koreth et al. 2009).

Sequential chemotherapy as a conditional regimen is used preceding HSCT and consists of a combination of anti-leukaemic and immunosuppressive therapies. In order to prevent graft-versus-host-disease (GvHD) immunosuppression therapy is essential and comprises of immunosuppressant drug treatment. There are two forms of GvHD, the most common is acute GvHD which can occur as early as a week following HSCT and takes place when alloreactivity between donor derived T-cells and the recipient's antigens. Chronic GvHD can take place 3 months+ following HSCT and is thought to involve alloactivation of donor CD4 and CD8 T-cells, although this mechanism is not yet fully understood (Kassim and Savani 2017).

Post-transplant (MRD) and chimerism status of the patient are monitored to assess graft success, although neither provide definitive guidance on whether a patient will relapse. Treatment with donor lymphocyte infusion (DLI) administered as a boost of immune cells from the donor can help to control relapse and potential leukaemic blast resurgence. Weaning off immunosuppressive therapy takes place following HSCT and monitoring of patient for signs of GvHD (Tsirigotis et al. 2016).

HSCs used for transplant are collected from either BM or more commonly peripheral blood (using GCSF mobilization treatment), and intravenously transplanted into the

the recipient where the cells then 'home' into the niche through the sequential activation of a variety of adhesion molecules (Podesta 2001).

Within the BM-niche, CXCL12 along with intercellular and vascular cellular adhesion molecules 1 (ICAM/VCAM) activate CXCR4+ progenitors which leads to their adhesion to endothelial cells. Cells which express insufficient CXCR4 detach and return to the bloodstream. CXCL12 and macrophage inflammatory protein-1 (MIP-1) then activate the binding of CD34+ cells into the extracellular fibronectin matrix of the BM niche via VLA-5 and VLA-4 integrin receptors (Kollet et al. 2001). The final stage of homing involves the migration of stem cells into the BM- niche where they interact with supporting cells, adhesion molecules, CXCL12 and growth factors. MSCs have been identified as an important contributory factor for a successful HSC engraftment. Several clinical trials have used MSCs to reduce aplasia post chemotherapy, prevent graft versus host disease (GvHD) and prevent overall transplant failure (De Luca et al. 2017), although the mechanism of action for this support is largely unknown.

The homing of the transplanted cells results in a depletion of progenitors and so only a small percentage of the recipient's stem cell pool is made up of these. The transplanted stem cells divide slowly but should generate enough progenitors to repopulate the host haematopoietic system to normal levels within 2 years of stem cell transplant (Lanzkron et al. 1999; Mahmud et al. 2001).

AML patients that relapse after transplantation have a poor prognosis (Figure 1.3), although current studies have shown that if treatment is started at molecular relapse rather than clinical relapse the results are more positive, however this requires regular monitoring of a suitable MRD marker which is not always present for all patients (Bejanyan et al. 2015).
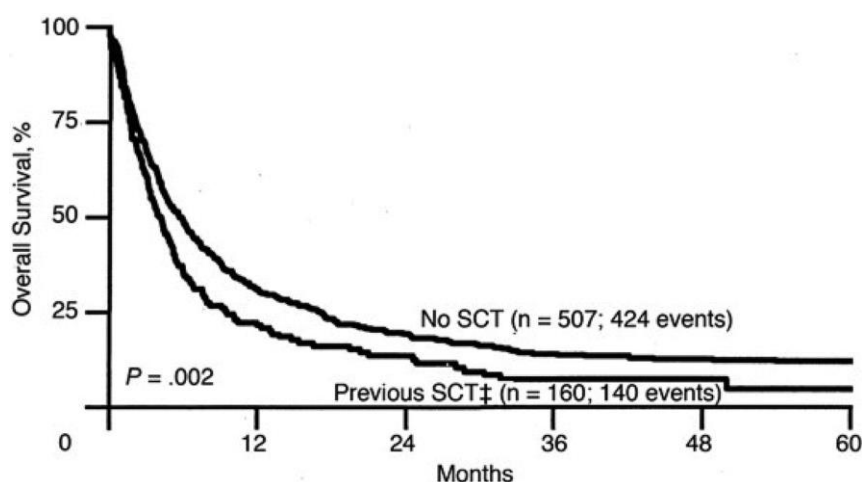
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Treatment for relapse of AML after transplant has one of two aims, to prevent leukaemic activity usually in the form of chemotherapy or enhance the immunological graft-vs-leukaemia (GvL) effect usually in the form of donor lymphocyte infusions (Orti et al. 2017; Stone et al. 2017).

Another possible treatment following relapse is a second stem cell transplant, but this is normally only suitable for younger patients due to the severe effects on the body. A recent study on AML patients showed that the 2-year OS rate after a second stem cell transplant was only 25% (Christopeit et al. 2013).

1.6 The bone marrow microenvironment as a driver of AML

During leukaemogenesis AML cells invade the BM-ME disrupting normal haematopoiesis and impeding the maturation of HSCs residing there (Morrison and Scadden 2014) (Figure 1.4)
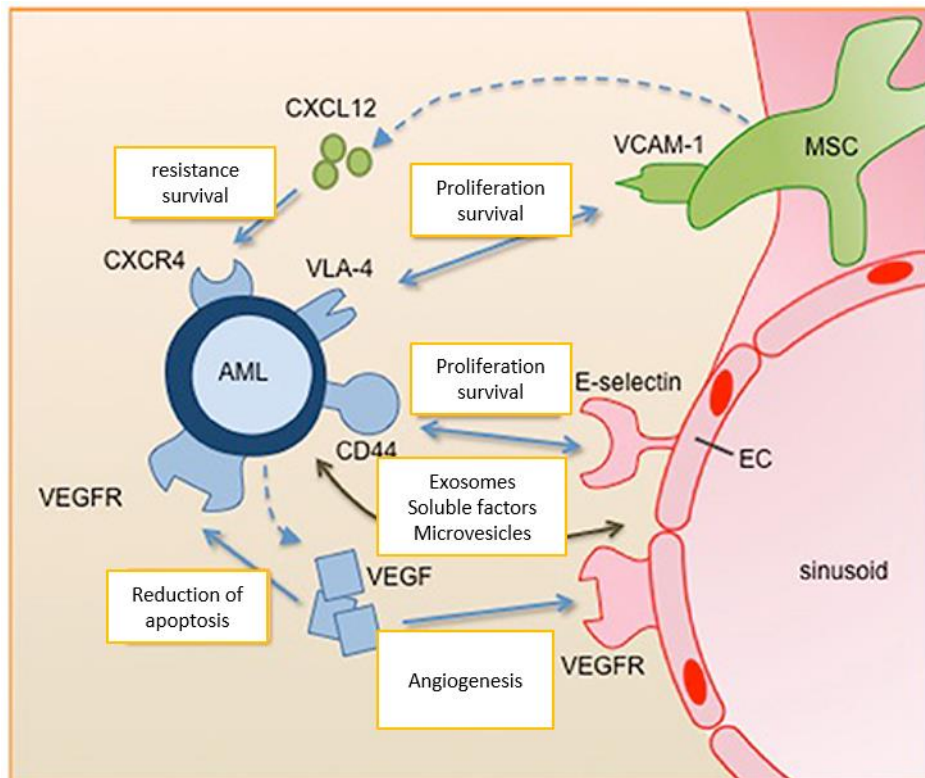
Figure 1. 4 Overview of cellular interactions that take place within the bone marrow microenvironment (Figure adapted from (Behrmann et al. 2018).

Once initiated, the LSCs manipulate the niche to their advantage allowing LSC expansion and chemoresistance before eventually remodelling the bone marrow microenvironment to produce a 'leukaemic niche' increasing the hypoxic environment which favours LSC survival and proliferation over normal haematopoiesis (Lane et al. 2009) (Figure 1.5). LSCs behaviour within the BM-niche is also modulated by interactions and signals with the various cell types that reside there such as endothelial, osteoblast and mesenchymal stem cells (Tabe and Konopleva 2015).
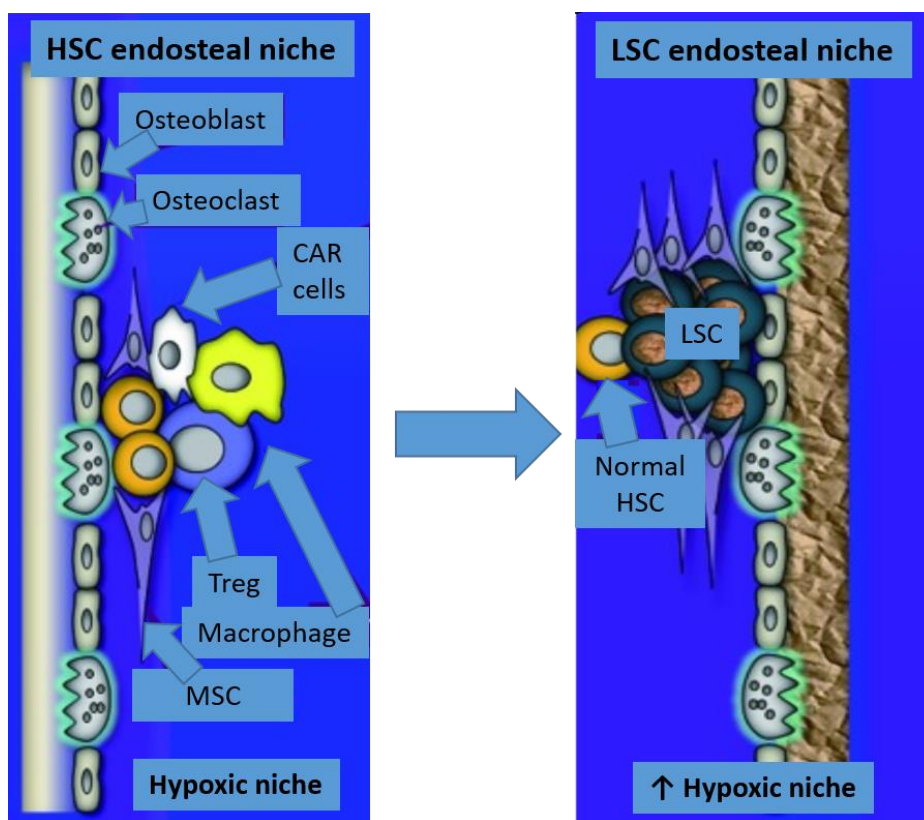
Figure 1. 5 Summary of differences between normal HSC endosteal niche compared to malignant LSC favourable endosteal niche. (Figure adapted from (Zhou H-S 2016)

Many studies suggest that not only does the BM-niche contribute to the acceleration of malignancy but that cellular and molecular changes can directly induce leukaemia. Two landmark studies by Walkley et al showed that genetic changes to the heamatopoietic and stromal cells of the BM-niche in the form of retinoblastoma gene deletion gave rise to myeloid disorders and showed that these genetic alterations within the niche were essential for disease initiation (Walkley et al. 2007). Studies have shown that dysregulation within osteolineage cells of the haematopoietic stem

cell niche can lead to the induction of myelodysplasia and secondary leukaemia (Raaijmakers et al. 2010). Extensive work carried out on murine models suggests that changes within the BM-ME can directly cause haematological abnormalities. Neoplastic changes causing disruption within osteolineage cell specific Dicer1 led to the development of AML, and those mice with osteoblast specific constitutively active β-catenin led to disruption of the Notch signalling pathway leading to AML (Kode et al. 2014).

These studies demonstrate a direct causal role for many of the cellular components that make up the BM-niche in the development of a number of myeloid diseases. The most commonly deregulated mechanisms implicated are Notch and Wnt signalling pathways and an increased production of pro-inflammatory cytokines (Schepers et al. 2015).

MSC's have been recognised as playing a major role in the leukaemic malignancy process, they increase the expression of several key niche factors that selectively support growth of LSC over HSC. VCAM-1 typically expressed on MSCs associates with CXCL12 which expresses CXCR4 on the cell surface of the leukaemic blast leading to LSC resistance and survival advantages (Jacamo et al. 2014). AML cells have been shown to block MSC differentiation into mature osteoblasts and instead induce the production of an osteogenic niche which acts as a sanctuary for LSCs and becomes inhospitable for normal HSCs (Battula et al. 2017). It has also been reported that MSC quiescence within the niche is disrupted by AML cells which reduce the density of the nerve network that is critical in order to maintain MSC in a state of quiescence and subsequent osteoblast differentiation and HSC survival (Yamazaki et al. 2011).

The BM-ME hosts a number of immunological cell types some of which are affected by the presence of a leukaemic niche. A reduction in numbers of T-cells and NK-cells is seen alongside an increase in Treg cells, thought to be instigated through the activation of immune markers, for example PD-1 whose expression levels are seen to correlate with AML development (Zhang et al. 2009). Macrophages associated with AML are also seen in higher numbers within the leukaemic niche helping to support their advancement (Winkler et al. 2010b).

Endothelial cells have been shown to aid the adherence and retention of AML cells within the BM-niche via the expression of E-selectin on their cell surface which AML cells recognise and readily adhere to. Endothelial cells also secrete the ligand VCAM-1 which interacts with the receptor VLA-4 on the AML blast cell providing another

potential site of anchorage (Becker 2012). Endothelial cells express VEGF recognised by VEGFR on AML cells which is a pro-angiogenic factor and an important contributing factor to increased leukaemogenesis (Fiedler et al. 1997).

VEGF expression by AML blasts induces neo-angiogenesis, this is known to increase within the BM-niche at diagnosis, recovering to normal levels at disease remission. Microvessel density (MVD) is a measure of angiogenesis and is used as a prognostic factor for patients, a higher MVD is associated with poor prognosis and increased risk of relapse (Schepers et al. 2015).

Within the BM-niche leukaemic cells secrete VEGF and other angiogenic factors upon integration into blood vessel walls, the release of growth factor angiopoietin (Ang) in combination with VEGF causes destabilization, degeneration and sprouting of blood vessels and which leads to increased angiogenesis and increased proliferation of malignant cells.

The role of fibroblasts in AML development is unclear but research has shown that an increase within the BM-ME has a protective effect on AML blasts (Zhai et al. 2016). A possible mechanism is via the release of the ligand MMP2 which is recognised by the receptor EMMPRIN on the AML blast and has been shown to lead to a reduction in apoptosis of AML cells (Gao et al. 2015). Fibroblasts make up a large proportion of the cells within the BM-niche but their function within the leukaemic niche is still not fully understood. Fibroblasts can produce extracellular matrix fibres and secrete cytokines and chemokines, and studies have shown that fibroblasts are involved in mechanisms of chemoresistance in solid tumours and AML, but it is not yet clear how this is achieved (Shirai et al. 2009; Zhai et al. 2016).

1.7 Bone marrow niche-mediated survival of leukaemia stem cells

Residual LSCs in the BM-niche are thought to play a large role in disease relapse and due to the poor prognostic outlook for relapsed AML patients, the interactions between LSCs and the BM-niche are thought to be an important therapeutic focus (Chiarini et al. 2016).

It is thought that the BM-niche mediates resistance to chemotherapy via several possible mechanisms, soluble factor-mediated drug resistance (SM-DR) consisting of the secretion of small molecules by osteoblasts, endothelial and stromal cells or cell adhesion-mediated drug resistance (CAM-DR) which is direct contact mediated by adhesion factors.

The direct interaction of AML blasts with the surrounding stromal supportive cells has been shown to increase their survival and offer therapeutic protection. AML induced changes of cytokine/chemokine secretory profile of the stroma has been shown to mediate the expression of receptors functioning as homing and protective devices for AML blasts (Sansone and Bromberg 2012; Civini et al. 2013).

Chemosensitivity within the BM-niche is mediated by cross talk between CXCL12 and CXCR4 (Ponomaryov et al. 2000). CXCL12 is a powerful chemoattractant for both HSCs and LSCs and CXCR4 activation has been shown to mediate the migration of AML cells beneath BM stromal cells. Malignant cells use CXCR4 to aid access to the protective niche which normally houses HSCs, and gives protection to drug treatment and encourages cell expansion (Burger et al. 2003). Studies have shown that upregulation of surface CXCR4 on AML cells creates a possible mechanism for drug resistance, the use of a CXCR4 small molecule inhibitor called AMD3465 successfully increased the sensitivity of AML cells to chemotherapy in vitro and in vivo. It is thought the inhibition of the CXCR4 pathway disturbs cell-matrix communication and encourages malignant cells to move from the quiescent sanctuary of the endosteal niche, and increase the chemosensitivity of the leukaemic cells (Andreeff et al. 2006; Zeng et al. 2009).

In vivo dynamic imaging has revealed that LSCs home and create vascular niches in cranial BM vasculature (Sipkins et al. 2005). Cells of the BM-niche secrete a variety of soluble factors some of which help regulate and retain LSC cells. CXCL12/SDF-1 is secreted by MSCs and binds to CXCR4 on leukocytes helping to regulate their transportation during normal hematopoiesis. CXCR4 expression on LSCs has been shown to bind to SDF-1 expressed by vessel endothelium and instigating LSC homing to the vascular niche (Sipkins et al. 2005). During chemotherapy it was observed that LSCs increase their expression of CXCR4 leading to an increase in survival and conferring resistance to therapy. Further confirmation of this mechanism was seen when CXCR4 was inhibited in AML cells and resulted in an increase in therapeutically induced apoptosis (Peled and Tavor 2013). Studies on CML have shown that the controlled release of soluble factors by BCR-ABL+ CML cells which included the decrease in CXCL12 and increase in GCSF, led to the movement of HSC cells out of the BM-niche and into the periphery, leaving it free for LSC monopoly (Zhang et al. 2012).

B-lymphoma cells have also been shown to produce soluble factors in the form of FGF4 which signals through FGFR1 on endothelial cells within the BM-niche, this

leads to an increased expression of Notch ligand Jagged1 which goes on to activate the Notch2 expressed on B-lymphoma cells leading to an increase in their proliferation (Cao et al. 2014).

Osteoblasts have been shown to play an important role in resistance to therapy in AML through both SM-DR and CAM-DR. Studies show that osteoblasts protect both CXCL12-expressing cell lines and primary AML cells from CXCL12-induced death via a SM-DR mechanism (Kremer et al. 2014). HSCs and LSCs are both known to tightly adhere to osteoblasts on the BM surface or stroma via a combination of integrins, cadherins and fibronectin and osteopontin, these interactions encourage cell mobilization and homing to the BM-niche leading to increased drug resistance (Kremer et al. 2014).

The differentiation of mature osteoblasts is blocked by LSCs inhibiting the sympathetic nervous system and inducing the secretion of CCL3. This leads to lowering osteocalcin levels and the construction of a pro-osteoblastic niche which contains high numbers of osteoprogenitor cells needed for LSC expansion alongside a reduction in normal haematopoiesis (Yamazaki et al. 2011; Frisch et al. 2012).

Osteopontin (Opn) is secreted by the osteoblasts within the BM-niche and is known to play a key role in maintaining cell quiescence, invasion and MRD. Studies have shown a correlation between increased expression of Opn and poor prognosis in AML (Liersch et al. 2012). It is thought that leukaemic cells expressing and secreting Opn are recruited to the BM-niche where they establish a Opn-rich region regulated by host-malignant cell exchanges via various integrins that interact with receptors expressed on Opn (Boyerinas et al. 2013). This protective malignant niche results in the expansion of the quiescent LSC population and resistance to chemotherapy.

Interactions between angiopoietin-1 (Ang-1) in osteoblasts and Tie-2 on HSCs give rise to many effects in cell adhesion, survival and quiescence (Reikvam et al. 2010). Tie-2 is a receptor tyrosine kinase expressed by both endothelial cells and AML cells, when it binds to Ang-1 it becomes phosphorylated and this activates the PI3-kinase – AKT signalling pathway (Bachegowda et al. 2016). Studies have shown the Ang-1/Tie-2 interaction brings about changes to the cell cycle, promoting cell cycle arrest in leukaemic cells, stopping cell division and maintaining self-renewal (Gomei et al. 2010).

AKT is known to suppress apoptosis, regulate proliferation and contribute to cancer progression and metastasis, it's activation results in HSC proliferation and it is thought could be involved in leukaemic transformation (Kharas et al. 2010). AKT can

phosphorylate FOXO transcription factors which aid the shuttle of FOXO proteins from cell nucleus to cytoplasm, this mechanism has shown to protect HSCs from noxious stresses, lower cell metabolism and help maintain HSCs in a quiescent state (Suda et al. 2005). Studies have shown that activation of the PI3K/AKT pathway in AML patients led to a favourable outcome, it has been hypothesized that this activation could promote entry of LSCs into the s-phase of the cell cycle making them more vulnerable to chemotherapy (Tamburini et al. 2007).

Endothelial cells are a major component of the BM-niche and due to their key positions within the vascular niche, LSCs migrate to these areas in order to develop vasculature (Cogle et al. 2014).

Adhesion molecules play an important role in successful engraftment of LSCs into the BM-niche. VLA-4 (very late antigen 4) is a cell surface ligand for VCAM-1(vascular cell adhesion molecule 1) found on MSCs and highly expressed on AML cells, this activates various pathways through NF-κB and helps mobilize and secure leukaemic cells into the niche and is also implicated in MRD (Papayannopoulou et al. 1995). The VLA-4/VCAM interaction within the niche encourages AML cells to adhere to surrounding endothelial cells which become activated by the release of VEGF-A which caused endothelial cells to form a chemoprotective barrier to AML cells this was further confirmed by studies which showed upon inhibition of VEGFR the chemosensitivity of AML cells increased (Poulos et al. 2014). AML patients which are VLA-4 negative are given a favorable outcome (Matsunaga et al. 2003; Jacamo et al. 2014).

The role of hypoxia within haematological malignances is controversial as there are many contradictory studies claiming hypoxia is both a positive and negative regulator of haematological disease progression (Drolle et al. 2015; Velasco-Hernandez et al. 2015). It is clear that activation of the HIF-1α pathway within the BM-niche results in increased angiogenesis and higher levels of cytokine secretion which results in an increase of the resistance of malignant cells to chemotherapy. Hypoxic microenvironment also plays a role in regulating the expression of CXCR4 leading to drug resistance (Fiegl et al. 2009). In summary, hypoxia plays an important *indirect* role in regulating drug resistance and disease progression.

LSCs have been shown to prefer to reside within the endosteal niche, this is typically a more hypoxic environment and LSCs are seen to localize and interact with surrounding cells including MSCs (Ninomiya et al. 2007). Just like the hypoxic endosteal niche seen in the normal BM-niche which houses HSCs, AML can bring

about genetic changes in MSCs enabling them to differentiate into osteolineage progenitors and start the modelling of a leukaemic niche (Raaijmakers et al. 2010). The hypoxic environment within the LSC niche becomes more extended than that seen in the normal niche which studies have shown is essential for LSC growth and survival (Benito et al. 2011).

LSCs play an important role in creating drug resistance in AML, they interact directly with the BM-niche and remodel it to create a protective niche that allows them to maintain a quiescent population and increases their self-renewal along with multilineage differentiation (Ishikawa et al. 2007). Relapse after chemotherapy is characterized by a more genetically heterogeneous and complicated LSC population (Ho et al. 2016).

Work carried out in ALL showed tunneling nanotubules (TNT) within the leukaemic niche which were used by LSCs to make contact with MSCs and induce secretion of various soluble factors which increased LSC survival and improved resistance to therapy. TNTs have also been observed in AML, in order to transport mitochondria between BM stromal cells, and AML blast cells within the BM-niche leading to improved AML cell survival. Stimulation of the transfer via the TNTs is driven by the generation of superoxide through NOX2 which stimulates the MSCs to carry out this mitochondrial transfer (Marlein et al. 2017). In vitro work has shown that the cholesterol lowering drug lovastin inhibited the growth of LSCs when co-cultured with MSCs but showed no affect when cultured alone, suggesting a link between metabolic activity and the support of AML survival within the BM-ME (Hartwell et al. 2013).

A recent study suggested that leukaemic cells can influence stromal response within the BM-niche in response to chemotherapy. The study involved the use of mice engrafted with human ALL cells and showed that following chemotherapy exposure a novel, but transient niche formed within the BM. This niche consisted of nestin1 leptin receptor MSCs surrounding ALL cells. The niche cells generated signals that confer resistance to drug treatment in the residing ALL cells, and some of these signals stimulated the release of pro-growth factors (Duan et al. 2014).

The adhesion molecule E-selectin is expressed on endothelial cells and binds to CD44 which is commonly expressed on LSCs creating an adhesion partnership within the BM-niche. Studies have shown that inhibition of CD44 and E-selectin leads to increased chemosensitivity of AML and a decrease in LSCs, signifying the protective nature of the BM-niche (Jin et al. 2006; IG et al. 2014).

There are several clinical trials aiming to address the barriers to chemotherapy created by LSCs within the BM-niche and reducing relapse and MRD in AML. Examples include; targeting CXCL12/CXCR4 axis using CXCR4 inhibitors to block the interaction of leukaemic cells with niche cells and reduce chemotherapy resistance, CXCR4/E-selectin inhibitor, more efficient dual function target, VLA-4 inhibitor to restore chemotherapy sensitivity and Hypoxia-activated prodrug (HAP) to address the hypoxic malignant BM-niche (Zeng et al. 2009; Layani-Bazar et al. 2014; Badar et al. 2016; Fogler et al. 2016)

1.8 Exosome biogenesis and mode of action

Extracellular vesicles are lipid-bilayer-delimited particles that are naturally released from cells and cannot replicate, they range in size from 20 – 1000nm. Diverse categorisation of extracellular vesicles has been proposed over the years, but this is a fast-changing field and consensus regarding nomenclature is not definitive. The following sub-types of extracellular vesicles are currently agreed (Ciardiello et al. 2016) :

- Ectosomes/microvesicles/microparticles (plasma membrane origin)
- Exosomes (endosomal origin)
- Apoptotic bodies
- Large oncosomes and other large extracellular vesicles
- Enveloped viruses
- Exomeres

For the purpose of this thesis I have chosen to refer to exosome as the extracellular vesicle sub-type that I believe to be relevant to my work.

Exosomes are the smallest within the family of extracellular vesicles and were first identified in 1983, and within the past ten years have become the focus of increasing scientific interest, consequently the field is constantly evolving as there is still much unknown (Pan et al. 1985). When exosomes were first identified they were initially believed to function primarily as waste disposal units, removing unnecessary proteins and other molecules from cells. It wasn't until the mid-1990's that exosomes were recognised as having an immunological function and since then numerous studies have implicated exosomes as having direct involvement in various biological processes. These processes include; inflammation, immune response and proliferation, stem cell maintenance and repair, tissue regeneration following injury and wound healing and within the development and progression of various diseases including most recently, cancer (Borges et al. 2013; M et al. 2017).

Exosomes are defined as 30-100nm in diameter and of endosomal origin, these two factors are the major defining features that set exosomes apart from other microvesicles (Kowal et al. 2014). The biogenesis of exosomes starts with the fusion of early-late endosomes forming multivesicular bodies (MVBs), the MVB membrane invaginates and forms intraluminal vesicles, a process which is partly driven by the endosomal sorting complex required for transport (ESCRT) (Hanson and Cashikar 2012). ESCRT consists of four protein complexes; Hrs, TSG101, STAM1 and VPS4B, all have been shown to support the inward budding process and the assemblage of exosome cargo (Henne et al. 2013). An ESCRT independent biogenesis has been observed but it is uncommon and thought to be driven instead by the presence of certain lipids (Babst 2011) (Figure 1.6)
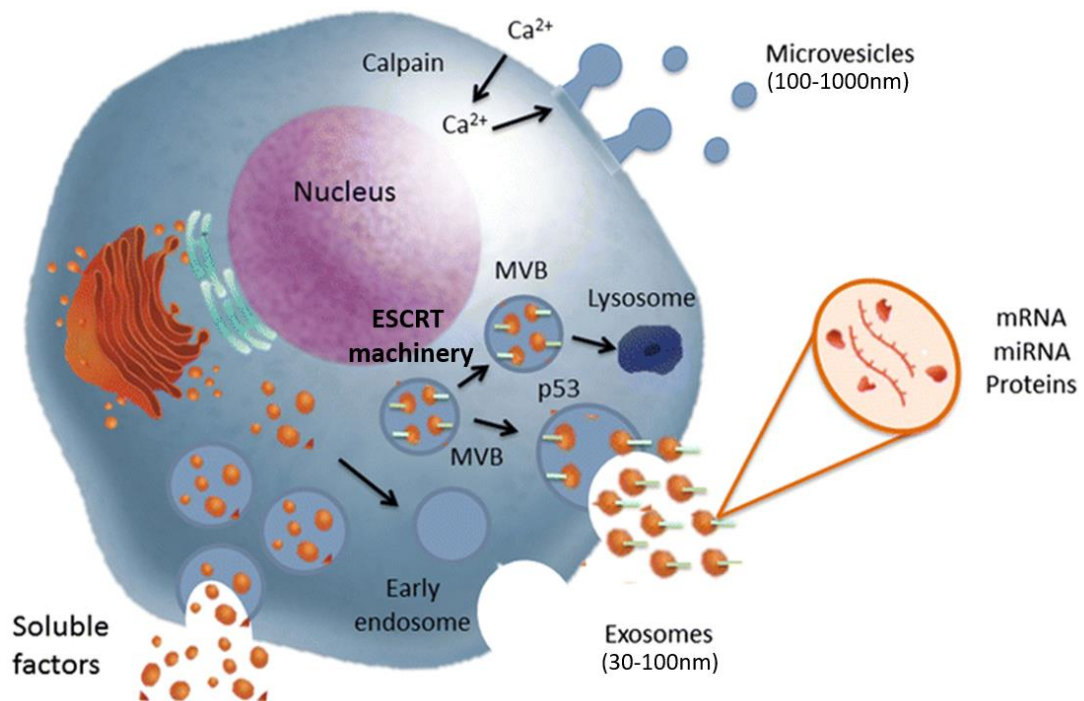
Figure 1. 6 Overview of the formation and biogenesis of exosomes and microvesicles within a cell (adapted from (Abreu et al. 2016).

When an exosome is formed the cell membrane becomes enriched with tetraspanins, these are proteins which are involved in many biological processes including cell adhesion, motility, invasion, membrane fusion, signalling and protein trafficking (Thery et al. 2009). The tetraspanins CD9, CD63 and CD81 are especially enriched on the membrane of exosomes and their presence is commonly used for identification. These proteins can also function as a selector of exosome cargo (proteins and miRNA), an aid to the binding and uptake of exosomes by target cells

and by presenting themselves to antigens and stimulating an immune response (Andreu and Yanez-Mo 2014).

It is thought that this selection process takes place using ubiquitination of proteins on the cell surface which act as a marker. Lipid rafts commonly observed on the membrane of exosomes can also function as cell signaling and sorting molecules (Stuffers et al. 2009).

The sorting of cargo into exosomes is suggested but not extensively proven so far to be selective, this is based on evidence that cargo carried by exosomes can differ from that of the parental cell. Information is delivered to precisely targeted recipients, which implies that the information carried must be programmed by the parental cell to be directed and specific (Ciardiello et al. 2016).

Once cargo has been sorted into exosomes the MVBs then either transfer to lysosomes where their content is degraded, or they are directed to the plasma membrane via an actin and microtubule cytoskeleton to dock and fuse (Hoshino et al. 2013). SNARE complex of proteins which consists of four SNARE protein helices at the plasma membrane are thought to facilitate the fusion of the MVBs with their target membrane and subsequent exosome release (Bonifacino and Glick 2004). Calcium levels within the cell also have an impact on the release of exosomes with a higher number of exosomes released in parallel with a rise in concentration of intracellular calcium, commonly seen within the bone marrow niche (Savina et al. 2003)

Rab GTPases 27A and 27B also play an important role within the late stages of exosome production, they help regulate vesicle budding, the transport of vesicles within the actin and tubulin cytoskeleton, and mediate fusion between the plasma membrane. It has been hypothesised that they are also involved in exosome secretion, but current studies show contradictory results therefore more work is needed to confirm this (Ostrowski et al. 2010). The transcription factor p53 a tumour suppressor gene commonly mutated within cancer patients, has also been implicated in exosome release. Studies have shown that activation of p53 gives rise to greater numbers of exosome release, and p53 knockout mice showed a severe reduction in exosome production. It is hypothesised that these exosomes contribute to the spread of malignancy by transfer of the protein TSAP6 which in turn leads to increased abnormal intracellular communication and induction of gene expression changes of non-diseased cells (Yu et al. 2006; Lespagnol et al. 2008).

Exosome content differs from cell to cell but always contains a combination of proteins, lipids and RNA. The main component of exosomes are lipids, they are

enriched in several different types e.g. cholesterol, as well as certain bioactive lipids e.g. prostaglandins and enzymes involved in lipid metabolism (Record et al. 2014). Protein content of exosomes depends on the cell of origin, they will share some membrane components and markers of the parental cell, but some physiological conditions and cell stimulation such as oxidative and thermal stress or chemotherapeutic agents can have an impact. An exosome which is derived from an antigen-presenting cell will be enriched in Ag-presenting molecules such as MHC-I and II complexes. Exosomes also contain key proteins involved in many important cell signalling pathways; NOTCH and Wnt-β-catenin pathways and interleukins, all important in regulating proliferation, differentiation and regulating immune response within the cell (Thery et al. 2009). Exosomes carry RNA in the form of mRNA, miRNA and lncRNAs which have all been shown to be functional in recipient cells, allowing their genetic modification by changing their translational profile (Skog et al. 2008). miRNA is generally seen at high concentrations within tumour cells and can differ significantly from parental cells. Work carried out using microarray analysis compared miRNA content of exosomes and their parental cell, results showed major differences. Certain mIRs were up to one thousand times more concentrated in exosome content, this indicates that there must be some miRNA preferential sorting mechanism, the process of which is not yet very well understood (Rabinowits et al. 2009; Hornick et al. 2015). The presence of certain sequence motifs and post transcriptional changes could indicate a possible sorting mechanism (Abels and Breakefield 2016) Figure 1.7.
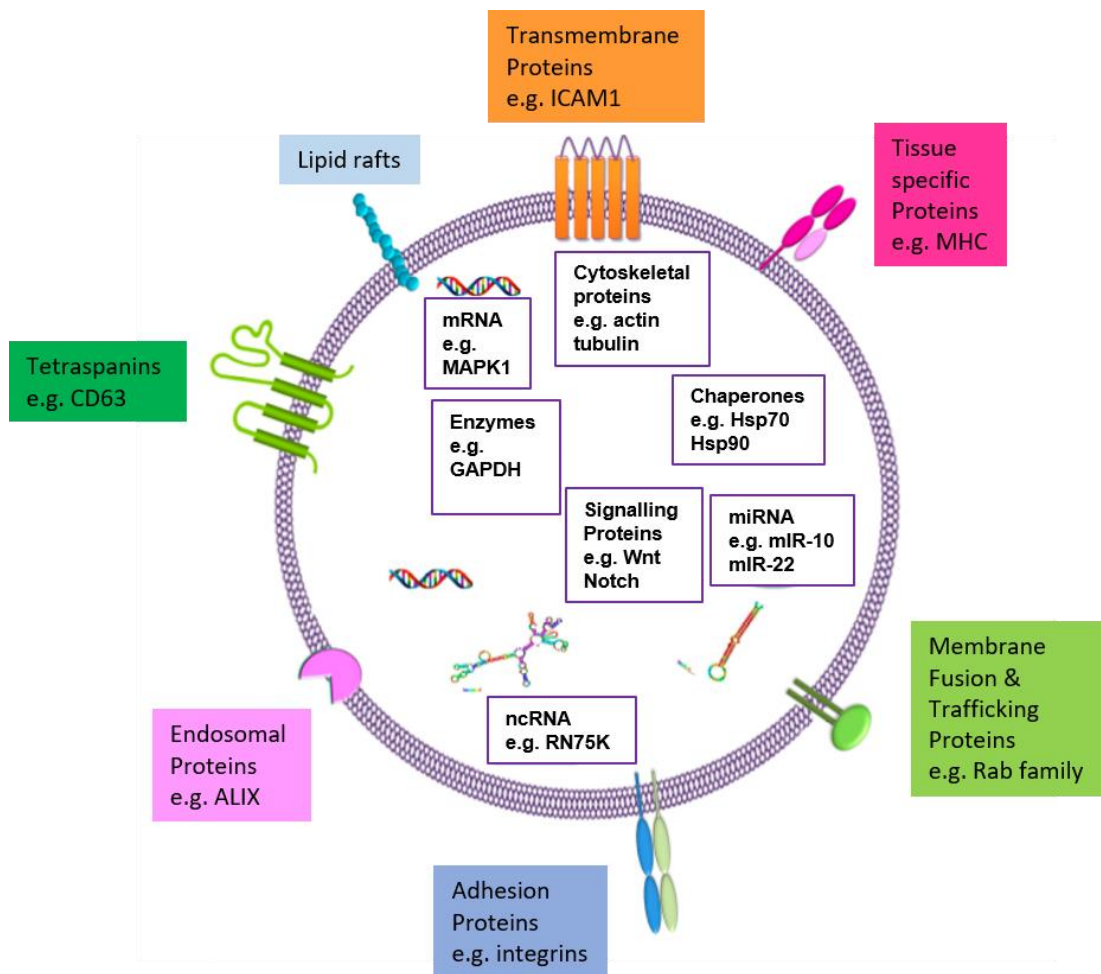
Figure 1. 7 The composition of an exosome showing both membrane bound and cytosolic molecular content. (Figure adapted from (M et al. 2017).

Uptake of exosomes is not fully established but endocytosis is the most commonly reported method, predominantly under the mediation of clathrin (Tian et al. 2014a). Phagocytosis (Feng et al. 2010), direct fusion with the plasma membrane (Parolini et al. 2009) or binding through exosomal adhesion molecules (Pan et al. 1985) are all possible alternatives but it's not clear whether this uptake is cell specific (Feng et al. 2010).

1.9   Exosomes and Cancer

Exosomes play an important role in several pathological processes within the human body and have been implicated in the progression of many diseases such as HIV (Teow et al. 2016), Parkinson's disease, Alzheimer's disease (Vella et al. 2016) and numerous inflammatory conditions (Lee et al. 2016). A significant interest is shown in

the extensive role exosomes play within multiple stages of cancer progression (Meehan and Vella 2016).

The way exosomes interact within cancer progression can be broken down into three key areas of association – the modulation of immune response, cross-talk within the malignant microenvironment and metastasis development (Ruivo et al. 2017).

Exosomes aid cancer immunosurveillance by presenting antigens to CD4+ and CD8+ T-cells via the MHC-I and II complexes on their cell surface. They also play a role mediating proliferation of HSCs and subsequent activation of NK cells which can go on to regulate an antigen specific immune response, leading to potential eradication of neoplastic cells (Yao et al. 2013). Exosomes also form an important part of the mechanisms involved in creating an immunosuppressive environment utilised by cancer cells to evade immunosurveillance via delivery of factors such as FAS ligands, that are capable of inducing apoptosis in the surrounding immune cells (Andreola et al. 2002).

Tumour-derived exosomes (TEX) can transfer information to recipient cells at local and distant locations within the body. Tumour cells which normally exist within hypoxic conditions such as the bone marrow, secrete large numbers of TEX. These communicate with other tumour cells or using either receptor/ligand signaling, plasma membrane fusion, phagocytosis or endocytosis, reprogramme recipient cells (King et al. 2012; Mulcahy et al. 2014). This reprogramming is thought to take place via the exchange of genetic material and signaling changes, which adapt the recipient cell RNA and create adjustments to the proteome (Mulcahy et al. 2014).

TEX are used to reprogram immune cells to escape host immunosurveillance and carry several immunoinhibitory molecules on their cell surface such as CD95 (FasL) and PD-L1 (Kim et al. 2005), TAAs (tumour associated antigens) expressed by the parental cell (Andre et al. 2002) and oncogenes or oncogenic proteins (Rak and Guha 2012).

TEX deliver signals to T-cells via receptor/ligand mediated signaling which causes an increase in intracellular calcium, which activates downstream signaling that changes the recipient cell transcriptome and it's function. Immune cells interaction with TEX varies, lymphocytes and monocytes very quickly internalize TEX, the method of transfer is thought to be dependent on the cargo being delivered (Muller et al. 2017). The result of reprogramming by TEX is an increase in growth of the parental tumour cells which is supported by a combination of cytokine and soluble factor release within the tumour microenvironment and periphery (Whiteside 2017).

TEX involvement in producing dysfunctional immune cells can also take place via negative signaling, and there is evidence to show that TEX signaling on recipient T-cells regulated the signal in a negative manner by inducing down-regulation of CD3-associated zeta chain (Taylor et al. 2003). TEX have also been shown to modulate transcription factors of recipient T-cells by activating the phosphorylation of STAT-5 in activated CD4+ T-cells, and inhibiting STAT-5 phosphorylation in activated CD8+ T-cells. As a result of this negative signaling, immune cells are restricted from eliminating the invading tumour cells (Whiteside 2013).

Dendritic cells have also been associated with anti-tumour immune responses, in vivo studies have shown that DC-derived exosomes cause activation of T-cells due to cross-presentation of tumour antigens. This shows that exosomes derived from different pathogenic parental cells can collectively bring about an immune response (Zitvogel et al. 1998)

Tumour-associated macrophages have shown to benefit from exosomes within key pathways associated with tumour development (Noy and Pollard 2014). Studies in breast cancer show that cancer derived exosomes cause activation of NF-κB in macrophages. This is known to bring about inflammatory response and release of various growth factors and cytokines, creating a permissive tumor microenviroment (Chow et al. 2014).

Cancer exosomes play an important role creating the tumour microenviroment by improving communication between the large network of supportive and malignant cells, helping to modulate and reprogram the surrounding cells to support and promote cancer cell proliferation. Tumour growth depends on access to nutrients and so vasculature within the tumour-micoenvronment is essential, exosomes have been shown to stimulate endothelial cells to secrete certain soluble factors which stimulate angiogenesis (Kucharzewska et al. 2013).

Exosomes play an important role in metastasis that depends on cell transport and invasive properties in order to survive and grow. Cancer cells use exosomes to induce invapodia formation, structures which help degrade the extracellular cell membrane allowing cancer cells to cross the endothelial barrier of recipient cells (Hoshino et al. 2013). Following invasion, cancer cells commonly go on to colonize a secondary site and direct this process through the expression of integrins (Hoshino et al. 2015).

Exosome involvement in haematological malignancies has not been as widely investigated as those in solid cancer, but they are believed to play a role in almost every aspect of the malignant haematological development. Plasma from patients

with a haematological malignancy contain much higher numbers of exosomes compared to that of normal origin (Szczepanski et al. 2011). In AML and CML these high numbers at diagnosis change once the patient begins therapy and a reduction in exosomal protein is observed in line with treatment, and in AML shows a correlation with leukaemic blast reduction. AML patients that achieved long term remission over two years saw plasma exosomal levels return to those seen in normal samples, reflecting the potential use of exosomes as a measure of disease and also an indicator of relapse (Hong et al. 2014b).

In AML, CML and MM exosomal derived nucleic acid content is linked to that of the parental cell, RNA from AML derived exosomes were shown to express AML prognostic markers NPM1 and FLT3-ITD (Huan et al. 2013). In miRNA from CML derived exosomes high levels of CD19 were shown signifying it's leukaemic B-cell origin (Ghosh et al. 2010). The miRNA and small RNA content carried by leukaemic cell line exosomes shows a greater level than that seen in the parental cells. miRNA of MM MSC-derived exosomes showed higher expression levels of oncogenic mIRs, and lower expression of tumour suppression mIRs compared to those from normal MSC-derived exosomes (Huan et al. 2013; Roccaro et al. 2013). This supports the theory that exosomes play an important role in the transfer of oncogenic material and the subsequent spread of haematological malignancy.

Exosomes have also been implicated in therapy resistance as described by a recent study using adoptive cell therapy (ACT) of activated NK cells as a form of immunotherapy in AML. This study produced disappointing results due to AML derived exosomes reprogramming NK-92 cells by delivering multiple inhibitory ligands to the receptors expressed on the cells, activating multiple suppressive pathways in NK92 which interfered with their anti-leukaemic function and reduced the overall therapeutic potential of the ACT(Hong et al. 2017) .

The therapeutic use of exosomes in recent years has become popular and they are currently being used in clinical trials as anti-cancer vaccines. Exosomes themselves have been identified as a potential inhibitory target as their numbers are often increased in correlation with severity of cancer. A certain component associated with the modulation, formation or release of exosome uptake is usually inhibited, an example of this is the Rab family of proteins implicated in exosome secretion. The disadvantage of such exosome inhibitors are their lack of specificity and critical role in all endosomal trafficking within cells leading to off target effects and high toxicity in patients (Datta et al. 2018). Exosomes are also being considered as a good way of

transporting drug or gene therapies into target cells as they are small and able to cross many biological barriers unimpeded. They commonly contain many adhesion associated proteins on their cell surface, and have low toxicity and immunogenicity due to their endogenous origin making them ideal for delivery (Tian et al. 2014b; Yang et al. 2015).

Due to their ubiquity within the human body and the correlation seen between circulatory levels and disease prognosis they have great potential use as a diagnostic biomarker for disease. Studies have shown that exosome biomarkers are known to perform better as a cancer diagnostic compared to biomarkers sourced from other liquid biopsy such as plasma, this could be due to the contents encasement within a protective barrier helping to withstand laboratory analysis techniques e.g. freeze/thaw and give a more stable readout (Wong and Chen 2019).

Current research in haematological malignancy has not explored the effect of stromal derived exosomes on cell to cell communication within the bone marrow environment and the preferential support provided to the AML cells that reside there. The potential mechanisms driving this support are unknown and forms the basis of this research.

1.10 Hypothesis

This study will investigate the hypothesis that exosome mediated communication is a contributing factor for the survival of residual AML blasts in the bone marrow niche. Monitoring of exosome content may identify molecular changes that have a functional affect in AML patient samples that could potentially be used to detect disease progression.

1.11 Study Aims

Within haematological malignancy the bone marrow microenvironment is a key player in mediating chemotherapy resistance and providing a sanctuary site from which leukaemic cells can evade chemotherapy, suppress immune response, produce disease resurgence and relapse.

Currently, residual disease within AML is unlikely to be overcome by pharmacological drug intervention and BM-transplant is still the most effective form of treatment for poor-risk patients, unfortunately there is still a high rate of relapse within this patient sub-set and subsequently this unmet clinical need forms the basis of my research.

AML patient samples are heterogeneous in nature but currently the associated risk factors such as molecular profile, FAB type and drug response at diagnosis do not fully explain the disparity observed between patient response post-transplant, making it difficult for clinical decisions to be made regarding patient treatment going forward. Bone marrow transplant is a complicated procedure with many factors influencing patient outcome. Serial sampling of patients in order to monitor changes that precede relapse rather than a comparison between patients, could potentially help overcome heterogeneity issues and identify targets linked to increased disease progression and potentially in the long term aid clinical decisions for patients that respond unfavorably post- transplant.

Exosomes secreted by the MSCs of the BM-niche are known to have an important role as message delivery vehicles but the impact they have in mediating cross-talk following post-BMT in AML is relatively unknown.

The aim of this project is to identify functional behaviours and potential biomarkers through MSC derived secretory profiling of exosomes, cytokines and miRNA from a range of disease stages

The aims are as follows:

1. To isolate AML-derived MSC cultures and quantify exosome production across a range of normal and disease states.

2. To characterise exosome contents at the miRNA and protein level and compare disease stages.

3. To assess the functional effects of MSC exosomes on AML cell within a stromal co-culture model.

2 Chapter 2. Materials and Methods

2.1 Composition of Stock Solutions

All reagents and plasticware were obtained from Sigma Aldrich UK Ltd (Dorset, UK) or Fisher Scientific UK Ltd (Loughborough, UK) unless otherwise stated.

2.1.1 Western Blotting Reagents

Cell lysis buffer (stock): 1.5ml 5M NaCl, 1ml 1M Tris buffer (pH 7.4), 5ml Glycerol, 0.5ml Igepal NP40, 1ml 0.5M EDTA, 5ml 1M NaF, 30ml ddH2O

Cell lysis buffer (working): 1 MiniComplete EDTA free tablet (Roche) dissolved in 1ml ddH2O, 8.7ml lysis buffer stock, 300µl 0.1M Sodium orthovanadate (New England Biolabs, Massachusetts, U.S.A), 1% Triton X-100

Running buffer: 950ml ddH$_2$0, 50ml 3-(N-morpholino) propane sulphonic acid (MOPS) running buffer (Invitrogen Ltd, Paisley, UK)

Transfer buffer: 100ml methanol, 1ml NuPage antioxidant, 50ml transfer buffer (Invitrogen Ltd, Paisley, UK), 849ml ddH$_2$0

Blocking buffer: 5% and 2% w/v TBS-T, milk powder

TBS-T: 10ml 1M Tris (pH 7.4), 20ml 5M NaCl, 10ml 10% Tween-20, 960ml ddH$_2$0

2.2 Tissue Culture Reagents

MEM dilution medium: MEM, Penicillin/Streptomycin, 20µM/ml Heparin sodium (CP Pharmaceuticals)

Primary cell culture medium: IMDM: Dulbecco's medium, HEPES buffer, sodium bicarbonate, 200mM L-glutamine, 10% Foetal calf serum (FCS) (Labtech Int. Ltd., U.K), Gentamicin (50µg/ml)

HS5 culture medium: DMEM (high glucose),10% Glutamax, 10% FCS, Gentamicin (50µg/ml)

MV411 culture medium: IMDM medium, 200mM L-glutamine, 10% FCS, Gentamicin (50µg/ml)

KG1a culture medium: RPMI medium, 200mM L-glutamine, 20% FCS, Gentamicin (50µg/ml)

MSC culture medium: DMEM (low glucose) medium, 200mM L-glutamine, 15% FCS, Gentamicin (50μg/ml)

Flow cytometry staining buffer: 0.5% BSA, 1x PBS, 0.02% Na Azide

TBS: 0.01M Tris (pH 7.4), 20ml 5M NaCl, 970ml ddH20

7AAD staining buffer: 1μg/ml diluted in PBS

2.2.1   FCS serum batch testing

FCS contains a complex mixture of components which may vary according to origin, nutritive conditions and processing. Because of this natural variation it is necessary to do batch testing, this consists of assessment of the growth support level of the serum of commonly used cell lines.

The cell lines HS5, MS5, KG-1a, and MV411 were harvested when in log phase of growth and resuspended at 4-8 x10^5 cells/ml in medium supplemented with different FCS batches. Cells were seeded within a 96-well tissue culture plate and placed into 37ᵒC incubator for 48 hours. Cells were harvested and cell viability assessed using 7-AAD and flow cytometry (see section 2.6). Cell viability was compared between batches to check no significant difference exists.

2.3   Antibodies

2.3.1   Antibodies Used in Flow Cytometry

All flow cytometry antibodies were sourced from Biolegend Inc. (San Diego, U.S.A) and used at a concentration detailed in section 3.2.2.

2.3.2 Antibodies Used in Western Blotting

Antibody	Molecular Weight (kDa)	Dilution	Supplier
Primary Antibodies			
CD9	24	1:3000	Santa Cruz Biotech.
CD63	26 & 30-60	1:3000	Santa Cruz Biotech.
CD81	22-26	1:3000	Santa Cruz Biotech.
Endoglin	84	1:3000	Santa Cruz Biotech.
CD73	71	1:3000	Santa Cruz Biotech.
Thyl	25-37	1:3000	Santa Cruz Biotech.
HER/Neu	185	1:1500	Santa Cruz Biotech.
ICAM-1	85-110	1:3000	Santa Cruz Biotech.
VCAM-1	110	1:3000	Santa Cruz Biotech.
IGFBP-3	40/44	1:3000	Santa Cruz Biotech.
TIMP-1	23	1:3000	Biolegend
LEPTIN	18	1:3000	Biolegend
ALIX	95	1:3000	Biolegend
ERK	42-44	1:5000	Biolegend
GAPDH	37	1:5000	Santa Cruz Biotech.
Secondary Antibodies			
Anti-Rabbit IgG		1:25000	VWR
Anti-Mouse IgG		1:25000	VWR

Table 2. 1 Summary of antibody details used in western blot and ELISA analysis

2.3.3 Antibodies used in ELISA assay

ALIX, TIMP-1 and IGFBP3 antibodies used at 1µg/ml, supplier detailed above in Table 2.1.

2.4 Tissue Culture

The following experiments were carried out in class II laminar flow biological cabinets on work surfaces sterilised with 70% ethanol prior to commencing work. All materials used in tissue culture were either purchased sterile or filter-sterilised prior to use and contaminated waste was disposed of using an autoclave.

2.4.1 Primary cells – Freezing and Thawing

Bone marrow samples were obtained at the University Hospital of Wales and normal marrow donors were obtained from Welsh Blood Service under specific ethical approval REC: 17/LO/1566, IRAS project ID:231974. All patients gave informed consent in accordance with the declaration of Helsinki and excess material was stored for research purposes and samples were collected in EDTA vacutainer tubes. A

nucleated cell count was performed on a 40µl aliquot of sample diluted in 20ml Isoton II diluents using a Coulter Z2 Particle Count and Size Analyzer (Beckman Coulter). 6-7ml aliquots of patient sample were layered over equal volumes of Ficoll-Histopaque 1077 using a syringe with 0.8mm aluminium hub needle. Samples were diluted according to the baseline count with warmed MEM dilution medium and FCS to give a maximum of 100×10^6 nucleated cells per Ficoll gradient. Following ficoll separation, mononuclear cells within the interface were removed and this suspension was washed twice in dilution medium, and the monolayer pellet was re-suspended in 500µl MEM dilution medium and pooled in a universal container. A further nucleated cell count was then performed. Mononuclear cells, suspended in MEM dilution medium, were divided into 500µl aliquots in 1.8ml cryovials, to ensure a maximum of 100×10^6 cells per vial. 400µl FCS and 100µl dimethylsulphoxide (DMSO) was added dropwise slowly to each vial and swirled. Cryovials were then transferred to a freezing container half filled with propan-1-ol and placed at -80°C overnight to ensure controlled-rate freezing. Cryovials were then moved to liquid nitrogen for long-term storage.

Cells were recovered from liquid nitrogen and 20µl DNase (10µg/ml) was added to the vial followed by 900µl of sterile 0.45µm filtered FCS, cells were then transferred to a 37°C water bath for 3 minutes incubation. Vials were removed, swabbed with alcohol and carefully transferred to a universal container, a doubling volume of IMDM complete medium was then added dropwise over three minute intervals in triplicate to prevent osmotic damage up to a volume of 14ml, cells were then centrifuged at 1000rpm for 10 minutes prior to resuspension in MEM complete media at a concentration of 5×10^6/ml for further analysis.

2.4.2 Cell Viability Assessment

Prior to spinning, an aliquot of cells was removed for counting, mixed 1:1 volume with 0.4% w/v Trypan Blue viability stain and cells counted by light microscopy using Fast-Read Disposable Counting Chambers (Immune Systems Ltd), where live cells exclude the Trypan blue stain and dead cells stain blue and a minimum of 200 cells counted. Cell viability was expressed as a percentage of live cells from total number of cells:

$$\text{Cell Viability} = \left[\frac{\text{Live cell count}}{(\text{Live + dead cell count})} \right] \times \text{Dilution factor}$$

2.4.3   Cell culture maintenance

MSC vials were thawed as previously outlined in 2.4.1 and allowed to form stroma typically taking 4-6 weeks to reach confluence following a 24 hour recovery period where any AML cells still in suspension would be removed (Figure 2.1) Medium change was carried out once a week by removing half medium and replacing with fresh. Cells were split once confluent by removing medium, washing with sterile 1x PBS, 5-7ml of Accutase added to a T75 tissue culture flask and placed in 37ºC incubator for no more than 10 minutes in order to remove cells. Cells were then spun at 1200rpm, supernatant removed, pellet resuspended, and cells counted before seeding into fresh tissue culture flask.
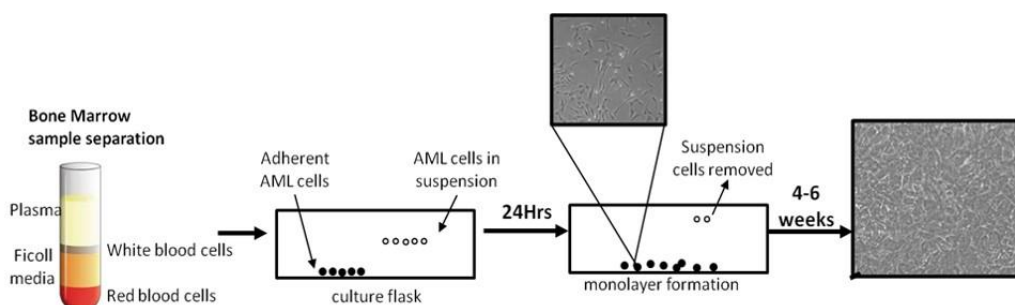
Figure 2. 1. Overview of primary AML derived MSC monolayer cell culture production.

HS5 cells were not used above passage 4 due to acquisition of culture associated genetic and differentiation changes. HS5 cells form a stromal monolayer quickly post-thaw and harvest of cells is carried out by first washing the cells with sterile 1xPBS after removing medium and then adding 5-7ml Trypsin-EDTA to a T75 tissue culture flask and incubating in a 37ºC incubator for ~ 5 minutes. Cells easily dissociate from the base of the flask base by pipetting 10ml of medium into flask and swirling. Cells were then transferred to a universal tube and centrifuged at 1200rpm, then counted and seeded at 1×10^5 cells/ml to be used at 80-90% confluency the following day.

Primary AML cell growth is sample dependent, cells are given a 24-hour recovery period post-thaw and seeded at a density of 7.5×10^5 cells/ml for experimental assays. Cells are split when required and medium changed 1-2 times a week by removing supernatant, centrifuging at 1200rpm, pellet resuspended in fresh medium and reseeded in fresh tissue culture flask.

MV411 and KG-1a cells are split 1-2 times a week as outlined for primary material, they are seeded at 0.5×10^5 cells/ml for experimental use and kept at $1\text{-}10 \times 10^5$ cells/ml confluency for maintenance.

2.5 Exosome Isolation

2.5.1 Collection of exosomes

Exosome free medium was produced in advance and prepared by making up growth medium containing double serum concentration plus usual additions of L-glutamine and gentamycin. Medium was ultracentrifuged at 100,000 x g for 17 hours at 4°C, aspirated off leaving pellet and then passed first through a sterile filter of 0.4µm then secondly a 0.22µm filter. Medium could then be made up to the working concentration of serum using serum free medium and added to culture when cells were close to confluency. Cells were always washed with serum free medium before exosome free medium was added to remove any residual serum derived exosomes.

2.5.2 Ultracentrifugation exosome isolation

Exosome containing medium was removed from tissue culture flasks and pooled into 50ml falcon tubes and cells spun at 2000 x g at 4°C for 20 minutes, supernatant was removed placed into fresh tube and spun at 10,000 x g at 4°C for 30 minutes, supernatants could then be stored at -80°C until ready for next stage of procedure. Supernatant was transferred to a thin wall polypropylene tube (Beckman Coulter, High Wycombe, UK) and ultracentrifuged at 100,000 x g at 4°C for 60 minutes (SW-28 rotor, Beckman Coulter, High Wycombe, UK). Partially purified exosome pellet was underlain with 4ml of 30% sucrose/D_2O (density of 1.2 g/ml) and ultracentrifuged at 100,000 x g for 75 minutes at 4°C. Approximately 2 - 3ml of the central base of the sucrose cushion solution was collected and diluted in excess sterile 1 x PBS. The exosomes were pelleted by ultracentrifugation again at 100,000 x g for 70 minutes at 4°C and pellets were resuspended in 50µl of sterile 1xPBS and stored at -80°C, until required for further experimental use.

2.6 Flow Cytometry

Flow Cytometry was performed using a Becton-Dickinson Accuri C6 cytometer coupled to a Dell Optiplex 765 Personal Computer running C-Flow Plus software for data acquisition and analysis.

2.6.1 Immunophenotyping

1×10^5 MSC cells were detached using accutase to preserve cell surface markers and washed before being placed in a universal container and centrifuged at 1200 rpm for

5 minutes, supernatant was removed and re-suspended in 40µl of ice cold staining buffer and 20µl of this suspension was placed in duplicate in a 96-well V-bottom plate. 10µl of antibody (see Table 3.2) were added to each well to give a total volume of 50ul per well. In the second well 10µl of the relevant anti-IgG1 isotype control for APC, PE and FITC was added. The plate was covered and vortexed gently and incubated at 4°C for 30 minutes. 150µl of cold staining buffer was added to stop the reaction and the plate was centrifuged at 1200 rpm for 3 minutes and the supernatant aspirated. Cell pellets were re-suspended in 50µl 1ug/ml 7AAD/FACS buffer for viability staining and this suspension was transferred to flow tubes for analysis where a minimum of 10,000 live events were recorded. Using C-Flow software, live cells (7AAD negative) were gated and CD45 was used to determine myeloid, lymphocyte and stromal fractions present at each passage with addition specific markers for target populations quantified using % positivity.

2.6.2 Cell proliferation assessment

Cells were harvested following assay incubation and if co-cultured with HS5 cells these were harvested separately. Cells were centrifuged in either a 15ml falcon tube or a V-well 96-well plate (depending on scale of assay) at 1200rpm for 5 minutes at 4ºC following 50µl addition of Absolute CountBright beads (ThermoFisher Scientific) per sample. Protocol was followed as above (2.6.1) using CD45 APC to differentiate HS5 cells from the rest of the cell population. At least 1000 bead events were acquired per sample and appropriate gating strategy used for analysis.

2.7 Western Blotting

2.7.1 Sample preparation and protein quantification

Exosome and whole cell cell lysates were prepared by adding 10µl of exosome preparation/cell pellet to 25µl of cell lysis buffer, mixed by vortex and incubated on ice for 30 minutes, samples were vortexed at 10 minute intervals during incubation. Following incubation samples were centrifuged at 1200rpm for 5 minutes to pellet debris and supernatant transferred to fresh 1.5ml Eppendorf tube and stored at -80°C until use.

2.7.2 Bradford Protein Assay

Bradford assay was set up in a MaxiSorp 96-well flat bottom plate (ThermoFisher Scientific), 10µl standards were prepared in advance using BSA and sterile water at 0,10,40,70 and 100µg/ml concentration and aliquoted in triplicate with 1µl of lysis buffer spiked into each standard to compensate for detergent interference. Bradford's stock solution was made up to working solution by diluting 1:1 with sterile water and

190µl added to all wells. The absorbance of the solutions was measured by reading the plate on a spectrophotometer at 590nm and protein content of exosome preparation calculated in µg/ml using the standard curve and appropriate dilutions.

2.7.3   Western gel electrophoresis

Unless stated separately, all pre-prepared reagents and materials used in western blotting including NuPage gels, LDS loading buffer, sample reducing agent, antioxidant, 3-(N-morpholino) propane sulphonic acid (MOPS) running buffer, 20x transfer buffer, Magic Mark XP Western Protein Standards and polyvinylidine difluoride (PVDF) membranes were supplied by Invitrogen Ltd (Paisley, UK). XCell SureLock Mini-Cell electrophoresis tank and XCell II Blot Module were also supplied by Invitrogen.

Samples were incubated in a heat block at 70°C for 10 minutes then quenched on ice for 1 minute before being loaded into pre-cast NuPage 4-12% Bis-tris gel (1mm 12 well) NP0322 (Life Sciences). Wells of the gel were previously washed in sterile water and running buffer with added antioxidant (200ml running buffer + 500µl NuPAGE antioxidant). Gels were run with MagicMarker molecular weight ladder (Life Sciences) at 200V constant for 50 minutes.

2.7.4   Western blot transfer

During electrophoresis a pre-cut nitrocellulose membrane (0.45um pore size) and filter paper sheets were pre-soaked in transfer buffer together with sufficient blotting pads to fill the transfer module. The gel was removed from the tank and after careful removal of the stacking gel a sandwich of blotting membrane, filter paper and pre-soaked blotting pads were constructed around the gel and the transfer module was filled with transfer buffer (Figure 2.2) The surrounding outer chamber was filled with ddH$_2$0 and run at 30V constant for 1 hour.
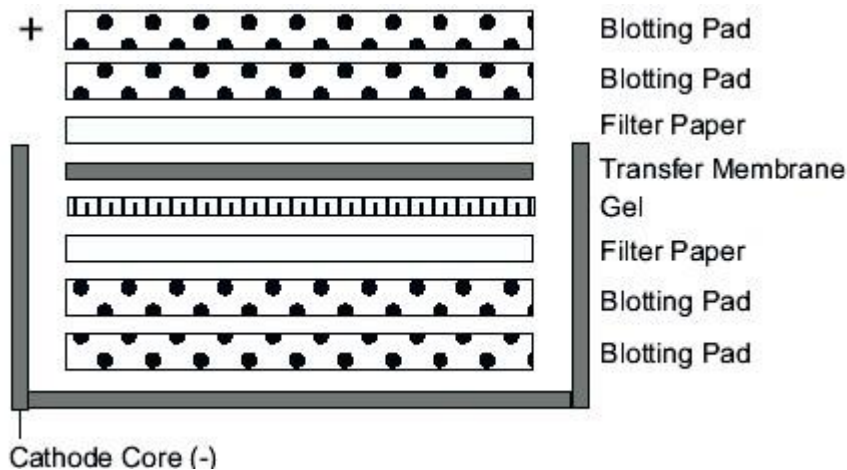
Figure 2. 2 Blotting apparatus for western blotting

2.7.5   Immunodetection

At the end of transfer the gel was removed and washed twice in 20ml ddH$_2$0 for 5 minutes. It was then incubated in 10ml 5% blocking buffer for 1 hour followed by washing in TBST for 30 minutes at 6 x 5 minute intervals. After this time the primary antibody was added, made up in 10ml 2% blocking buffer and diluted according to the manufacturer's instructions and incubated overnight on plate shaker at 2-8°C.

Following incubation, the membrane was rinsed twice in TBST and then washed at 5 minute intervals for 30 minutes, it was then incubated in rabbit or mouse secondary antibody at 1:25000 in 2% blocking buffer for 60 minutes. After this time the membrane was washed at 5 minute intervals for 30 minutes and developed with Chemiluminescent Substrate ECL Select (VWR, Pennsylvania U.S.A) for 5 minutes. Finally, the membrane was processed on the LAS-3000 imager for a period of time determined by the strength of the signal ranging from 1-60 minutes.

2.8   Luminex Analysis

2.8.1   Sample preparation

Exosome preparations were lysed using a cell extraction buffer (see recipe below) which was made in advance, aliquoted into 10ml tubes and stored at -20°C. Before use, one protease inhibitor tablet (Roche, IN, U.S.A) and 1mM PMSF was added and stored at 4°C for up to 4 weeks. 10µl of exosome preparation was added to 25µl of 2x Luminex cell extraction buffer and standard lysate protocol was followed as outlined previously (2.7.1).

2x Cell Extraction Buffer: 10mM Tris pH 7.4, 2mM Na_3V, 100mM NaCl, 1% Triton X-100, 1mM EDTA, 10% Glycerol, 1mM EGTA, 0.1% SDS, 1mM NaF, 0.5% Deoxycholate, 20mM $Na_4P_2O_7$.

Exosome samples prepared in this extraction buffer had to be diluted 10-fold to avoid interference within the Luminex run, I diluted all of my exosome lysates for Luminex analysis using the Luminex cell extraction buffer to equalise to a concentration of 20µg/ml, they were then diluted within the 96-well test plate to a final concentration of 2µg/ml.

2.8.2 Luminex analysis

For all of the following analysis Magnetic Luminex Assay: Human Premixed Multi-Analyte Kit (R&D Systems Inc., Oxford, U.K) was used. 104 cytokine and chemokine targets were multiplexed over three luminex bead array panels, these included several known MSC derived candidates which have previously been described (FLT3, GAS6, FGF2, EGFR, IL-8, TIMP-1, TGF-β, TNF, CCL3, MCP-1 (Huang et al. 2015; Corrado et al. 2016) and have suggested links to disease progression as well as other targets whose functional consequences are unknown within the post-transplant setting and cover a variety of biological processes including survival, differentiation, immunology, chemoattract and adhesion. All reagents were brought up to room temperature and standards were provided with the kit but varied in number depending on the analytes selected. Each standard was reconstituted with Calibrator Diluent, volume added was dependent on number of standards provided. Standards were multiplexed and a serial dilution was then carried out to produce a 3-fold dilution series using 100µl of standard and then 200µl diluent, tubes were mixed thoroughly between transfers and a calibrator diluent blank was included.

Diluted microparticle cocktail, biotin-antibody cocktail and streptavidin-PE were all prepared according to kit instructions. 50µl of sample and standard were added to designated wells of a 96-well plate and 50µl of microparticle cocktail was added on top, a foil lid sealed the plate and it was placed on a shaker set at 800rpm for 2 hours at room temperature.

After incubation a magnetic device was used to wash the plate with 100µl of wash buffer, leaving for 1 minute then removing and repeat this washing process three times 50µl of biotin-antibody cocktail was added to each well, covered with foil plate sealer and incubated on plate shaker set at 800rpm for 30 minutes.

Plates were washed as previously described and 50µl of streptavidin-PE was added to each well, sealed and incubated on shaker set at 800rpm for 30 minutes.

During this incubation period the Luminex Analyzer was set up by switching on to warm up then running calibration programme which uses highly purified recombinant human biomarkers to ensure the system is operating correctly and maintain data accuracy. Instrument settings were inputted, these included assigning the microparticle region for each analyte being measured.

Plates were washed as previously described and microparticles resuspended in 100µl of wash buffer to each well, the plate was incubated on shaker set at 800rpm for 2 minutes and then read using Luminex 200 Analyzer instrument.

2.9 Permeabilised Exosome ELISA Assay

Exosomes were diluted in PBS and bound onto a high protein binding ELISA strip plate (Greiner Bio-One) at a dose of 1µg/well and incubated overnight at 4°C. Wells were washed three times using a wash buffer (Kaivogen), to remove unbound particles and then blocking solution (1% BSA/PBS, w/v) was added for 2 hours, before washing three times. Radioimmunoprecipitation assay (RIPA) lysis buffer (Santa Cruz, Texas, USA) was added for 1 hour at RT, before being washed three times. Primary antibodies were added at 1µg/ml (2.3.3), for 2 hours at RT. After washing wells three times, the primary antibodies were detected by goat anti-mouse biotinylated antibody (Insight Biotechnology) (diluted in 0.1% BSA/PBS, w/v), at a 200ng/ml working concentration for 1 hour at RT. The wells were washed three times, before adding a europium-streptavidin conjugate (Perkin Elmer) in assay buffer (Kaivogen) for 45 minutes at RT. The wells were washed six times, before adding enhancement intensifier (Kaivogen) for 5 minutes at RT and signal assessed by TRF on a PHERAstar FS Microplate Reader (BMG Labtech).

2.10 Statistical Analysis

All standard statistical analyses were performed using Prism-6 Software V6.07 (GraphPad, San Diego, USA). Luminex data analysis (PCA and hierarchical charts) were carried out using Partek Genomics Suite (Partek Inc, Missouri, USA). All experiments were performed in triplicate. In experiments with more than two experimental groups, 1-way ANOVA was used with Kruskal-Wallis post-test to further investigate variability between groups. When comparing two experimental groups evaluation was carried out using Students t test for cell line experiments with parametric data spread and Mann-Whitney U (for non-parametric primary AML experiments) to compare ranks; a particularly robust analysis for data sets that

contain outliers. P-values less than 0.05 were considered significant and graphs depict mean +/-SEM three similar experiments unless otherwise stated.

3 Chapter 3: Isolation and Characterisation of Extracellular Vesicles

3.1 Introduction

3.1.1 Exosome definition and identification

Exosomes are the smallest sub-category of extracellular vesicles and have been implicated in numerous cancer studies as a major contributor within the cell to cell communication network acting as delivery vehicles of various soluble factors including miRNA, proteins and lipids known to aid pathological processes including malignant transformation. Due to their small size they have an advantage over the larger sub-category of extracellular vesicles as they are able to travel further and cross major biological barriers going relatively undetected by immunosurveillance as well as being more easily assimilated by acceptor cells, this has made them an ideal candidate for targeted gene/drug delivery, a popular clinical strategy in recent years.

Previous studies in AML have shown that plasma originating from diagnostic AML patients contains higher levels of exosomes compared to that of normal plasma (Hong et al. 2014a), however the content and number of exosomes released within AML patients at different stages of treatment and particularly in the post bone marrow transplant (BMT) environment has not yet been well characterized and forms the basis of my investigations.

Previous work has shown that cancer derived exosomes can contribute to the progression of cancer by aiding the intercellular transfer of protein, lipid and nucleic acid cargo within the malignant microenvironment. Numerous oncological studies have shown that malignant cells secrete higher numbers of exosomes compared to healthy cells and the cargo they transport also differs (Al-Nedawi et al. 2008; Skog et al. 2008; Luga et al. 2012; Peinado et al. 2012).

Exosomes harvested from urinary samples from cancer patients have been used to identify protein markers such as the EGFR pathway, α-subunit of GsGTP binding protein, resistin and retinoic acid induced protein which are absent in healthy individuals (Smalley et al. 2008). Pancreatic cancer patients have shown the presence of Glypican-1 (a cell surface proteoglycan) on exosomes derived from patient serum in both early and late stages of disease, which was absent in benign samples (Melo et al. 2015). Work on ovarian cancer has shown that when exosomes

from diseased patient plasma are compared to benign or normal, higher levels of exosomes were seen along with the presence of TGF-β1 and MAGE3/6 protein markers. In addition to higher exosome levels in diagnostic samples compared to normal, exosome increases are also observed with disease progression (Szajnik et al. 2013).

Research has shown that miRNA signatures for exosomes derived from cancer patients do not always correspond to that of the parental tumour cell (Nolte-'t Hoen et al. 2012), it has been suggested that the miRNA that exosomes carry is selected, sorted and directed by the parental cell to specifically targeted recipients (Ciardiello et al. 2016). Disease specific miRNA profiles derived from exosomes have been identified in a wide variety of cancers including colorectal, lung and breast (Roberson et al. 2010; Corcoran et al. 2011; Cazzoli et al. 2013; Ogata-Kawata et al. 2014). Disease progression and therapeutic response has also been linked to expression of miRs such as miR-21 which is overexpressed in exosomes originating from ovarian and cervical cancer cells and correlates with increased disease progression (Taylor and Gercel-Taylor 2008; Liu et al. 2014).

The nomenclature of extracellular vesicles is constantly being developed and refined within the research community, but the following two characteristics are universally accepted as exclusive to the exosome subcategory of microvesicles:

A. Particle within the size range of 30-100nm

B. Generated by inward budding of endosomal multivesicular bodies and release via fusion with the plasma membrane

Recent work using asymmetric flow field fractionation (AF4) technology which separates particles based on their density and hydrodynamic properties has identified a further sub-population of exosomes, large exosome vesicles (90-120nM) and small exosome vesicles (60-80nM) alongside an abundant population of non-membranous nanoparticles (~35nM) named 'exomeres'. Exomere profiling revealed the presence of various proteins involved in pathways such as glycolysis and mTOR signalling. The large and small exosomes showed the presence of lipid, protein, DNA and RNA species (Zhang et al. 2018).

Identification of exosomes using an associated protein marker is difficult, there is no single reliable marker as any cargo carried on the exosome membrane must have originated from the endosomal membrane, and anything inside would have come from the cytosol, meaning that even though the cargo might carry more of a certain

molecule within the exosome, it is not exclusive to it. Typically, a combination of markers is used, for example the tetraspanins (CD63, CD81, CD82) known to be enriched within the exosome membrane, proteins associated with ESCRT machinery (TSG101), cytosolic proteins associated with exosomes (GAPDH) and antigen presenting molecules (MHC) are all cellular components that make up the molecular composition of an exosome.

Characterisation of exosomes is an area of ongoing research which is required in order to standardize assessment criteria. Updates to guidelines and criteria for defining extracellular vesicles are published every four years by the International Society for Extracellular Vesicles, the most recent publication was 2018.

General characterization of extracellular vesicle preparations should follow the guidelines outlined by the Society of Extracellular Vesicles as follows:

1. Presence of at least three protein markers including:

    a) Transmembrane/lipid bound protein

    b) Cytosolic protein

2. Characterization of single vesicles preferably using both of the following techniques:

    a) Electron or atomic force microscopy

    b) Single particle analyzers

3. Quantification of vesicle preparation, of which there is no single preferential method, total protein and total particle number is most commonly used. Both components are not exclusive to vesicles, proteins are soluble, and particles can be aggregates so a measure incorporating both in the form of a ratio of protein: particle is recognised as the most reliable measurement of EV preparation purity.

3.1.2 Overview of exosome isolation and methodologies

Isolation of exosomes is difficult to achieve due to similarities in the biophysical characteristics between exosomes and extracellular vesicles within the same size range, most preparations will most likely contain a mixture of small EVs which is why some prefer to use the term 'small extracellular vesicles' instead of exosome. Many factors including cell type and environmental conditions can influence the content of

the exosome preparation and most current isolation protocols will produce a mixture of exosomes, extracellular vesicles of non-endosomal origin, lipid-based non-vesicular structures, lipoproteins and exomeres.

There are several different isolation techniques which can be used depending on the degree of exosome purity and concentration required, a summary of the frequently used methods is as follows.

1. Ultracentrifugation

This is the most commonly used technique to isolate extracellular vesicles and is considered the gold standard of exosome isolation due to the relatively minimal long term running cost and technical expertise needed, along with the low levels of contaminating vesicles present in the resulting prep. Different centrifugal forces sediment particles according to density, size and shape, the centrifugal force ranges from 100,000 to 120,000 x g. Initial low speed spins are used to separate out dead cells and debris, then a series of high speed runs between which the supernatant or pellet is resuspended until finally the exosomes are isolated and resuspended in appropriate buffer and stored. Alternative steps can be added to the ultracentrifugation protocol to increase the purity of the sample although this comes at the sacrifice of exosomal yield. A sucrose cushion step can be added onto which the sample is layered and following centrifugation, the exosomes in the sample separate out into discrete zones due to their different densities and can then be recovered by simple fraction collection. This step will eliminate contaminants such as non-specific proteins or large protein aggregates which are sedimented out but do not float on the sucrose gradient.

2. Size exclusion

Exosomes are separated based on their size or molecular weight and passed through a physical barrier using chromatography or filters, one of the popular exosome isolation techniques is ultrafiltration which follows the same format as standard filtration but with the additional use of applied force. Usually a 0.8µm filter is used initially to remove cell fragments then a 0.2µm filter is used to isolate exosomes. To enrich the exosome preparation further, filtering steps using low-molecular weight filters can be used. Although ultrafiltration is a much quicker process than standard filtration, it has since been reported that forcing particles through filters can lead to particle deformation which may affect their use in functional assays (Witwer et al. 2013).

3. Immunoaffinity Isolation

This isolation technique uses the presence of specific surface proteins associated with exosomes using antibodies to immune-enrich or deplete samples. Antibodies are usually associated with beads or other matrices and physical separation takes place following low-speed centrifugation or magnetic technique, there are several commercially available kits based on the concept of magneto-immunocapture. Immunological techniques are highly specific in their isolation of exosomes and in some cases are regarded as a superior method to ultracentrifugation although at the cost of much lower yields. Therefore, these methods are most beneficial when used in combination with other techniques in order to further purify and enrich the exosome preparation.

4. Polymeric precipitation

Exosome containing solutions are incubated with a precipitation solution containing water-excluding polymers, following overnight incubation the exosomes are precipitated out of solution and are further isolated using low-speed centrifugation or filtration. There are a number of commercially available exosome precipitation kits available and they are very quick and easy to use with minimal specialist equipment needed, results have shown high yields of RNA but a number of non-exosome contaminants such as proteins and polymeric materials have been seen to co-precipitate with the exosomes resulting in an exosome preparation which is highly likely to contain high levels of contaminating particles.

5. Microfluidics

The most recent development in exosome isolation, microfluidic devices are manufactured to use both biochemical and physical characteristics to isolate using electrophoretic and electromagnetic mechanisms. Microfluidic devices can trap exosomes and filter out unwanted proteins, larger extracellular vesicles and cell debris using immunoaffinity, size and density. The specificity of these devices can be enhanced using immunoaffinity capture within the microfluidics to isolate specific exosomes. The advantage of these devices is the low volume of input material and reagent needed and preparation time very low, although the application of these methods within biological fluids has not been extensively investigated so far (Li et al. 2017).

Table 3.1 summarises the pros and cons of the current most popular exosome isolation techniques.

Technique		Advantage	Disadvantage
Ultracentrifuge	Density, size, and shape-based separation	Reduced cost Low contamination risk Large sample capacity Large yield of exosomes	High equipment cost Labour-intensive protocol Potential damage to exosomes
Size Exclusion	Isolation based on size difference between particles	Ultrafiltration: Fast, no special equipment needed SEC: high-purity yield, exosome integrity and biological activity preserved, good reproducibility.	Ultrafiltration: low equipment cost, moderate purity and potential deformation of exosomes SEC: moderate equipment cost, long run time.
Immunoaffinity Capture	Isolation based on specific interaction between membrane-bound antigens and immobilized antibodies	Excellent specificity Highly purified exosome yield Possibility of subtyping	High reagent cost Low capacity and yields Only compatible with cell-free samples Tumour heterogeneity can confuse immune recognition
Precipitation	Isolation using water excluding polymers to alter solubility of exosomes	Easy to use No special equipment Easily scaled up or down	Co-precipitation of contaminants Long run time Requires pre-and post-clean-up.
Microfluidics	Microscale isolation using immunoaffinity, size, and density.	Fast Low cost Portable Easy automation	Lack of standardization Minimal clinical validation Low sample capacity

Table 3. 1 Summary of exosome isolation techniques

The isolation and characterisation of exosomes is an important area of research which is constantly being updated. In order to adequately facilitate research and potential clinical applications, development is necessary to successfully isolate, subtype and quantify total exosome populations. Choice of methodology will depend primarily upon both downstream applications and source material, these are important factors to assess before selecting a technique.

3.2 Methods

3.2.1 Culture of mesenchymal stem cells

According to the methods described in Chapter 2 MSC cultures were cultured and once reached confluency, adherent MSCs were detached using 5ml of TrypLE Express and incubated for no longer than 5 minutes in a tissue culture incubator. Once cells were detached and counted ~1-2x10^6 cells were frozen down for future use according to methods outlined in Chapter 2, (these cells were classified as passage 1 (P1)). 1x10^5 cells were set aside for immunophenotyping and the rest split into two T75 flasks, fresh growth medium added and expanded for 2 weeks. Cells were sub-cultured until confluent and the process repeated until passage 3 for use in subsequent assays. MSCs cultured further beyond P4-5 are known to induce genetic and differentiation culture-related changes (Bonab et al. 2006).

3.2.2 Immunophenotyping of stromal cultures

MSCs were collected following every sub-culture to passage 3, 1×10^5 cells were centrifuged at 1200rpm to form a pellet, supernatant removed and resuspended in appropriate volume of ice-cold staining buffer. Cell suspension was added to 8 wells of a 96-well V-bottom plate, centrifuged for 1200rpm for 3mins, aspirated and 50µl of each of the antibody cocktails shown in Table 3.2 were added to the appropriate well, mixed and incubated at 4°C for 30 minutes. The addition of 100µl of cold staining buffer ended the reaction, the plate was centrifuged at 1200rpm for 3 minutes and cells were resuspended in 100µl of 1µg/ml 7AAD solution in order to assess cell viability. Flow cytometry was used to analyse samples, a minimum of 5000 live events were recorded using the BD Accuri Cytometer. C-flow software was used to analyse samples using gating appropriate to the fluorescent labelling used within each antibody cocktail and expression levels of each target was quantified by mean fluorescence intensity.

	APC (FL4)	FITC (FL1)	PE (FL2)	7AAD (FL3)
1	CD45	CD44	CD90	+
2	CD45	CD14	CD34	+
3	CD45	CD29	CD73	+
4	CD105	CD45		+
5	CD71	CD45		+
6	CD45	CD31	Nestin	+
7	CD324	CD146		+
8	IgG	IgG	IgG	+

Table 3. 2 Antibody cocktails used for MSC phenotyping

3.2.3 Microscopic analysis of MSC cultures

In order to gain more insight into the effect of cell size and numbers of the MSC cultures on exosome production, MSC cultures where grown to 70% confluency prior to the addition of exosome free media. Multiple microscopic images were taken on

harvest using GXCapture-T software and ImageJ 1.52i analysis (http://imagej.nih.gov/ij) to generate cell number and cell area in µM^2 for each MSC culture layer across the same magnification

3.2.4   Collection and extraction of exosomes

Once stromal culture reached ~80% confluency, growth medium from flask was removed, cells were washed using serum free medium and replaced with either 15ml if using a T75 or 30ml if using a T175 tissue culture flask of exosome-free medium and placed back in the incubator for 5 days.

Two methods of exosome isolation were used to compare yields:

1. Commercially available immunoaffinity Exosome Isolation Kit (Miltenyi Biotec, UK)

2. Gold standard ultracentrifugation technique adapted from Thery et al 2006.

The isolation of exosomes using the Miltenyi Biotec Exosome Isolation Kit Pan uses MicroBeads which recognize the tetraspanin proteins CD9, CD63 and CD81. 2ml of exosome conditioned supernatant consecutively centrifuged at 300xg for 10 mins, 2000xg for 30 mins and 10000xg for 45 mins to remove dead cells and debris. This was then incubated with 50µl of MicroBeads at room temperature for 1 hour, during which µMACS columns were primed by attaching to the MACS magnetic stand and running 100µl of Equilibrium Buffer followed by three washes of 100µl of Isolation Buffer. The labelled exosomes were then loaded onto the primed µMACS column which was placed within the magnetic field of a µMACS Separator. Magnetically labelled exosomes are retained within the column while the unlabelled vesicles and cell components run through the column. Exosomes were eluted using 100µl of Isolation Buffer and flushed out using a plunger in the column. A summary of the protocol is shown in Figure 3.1.
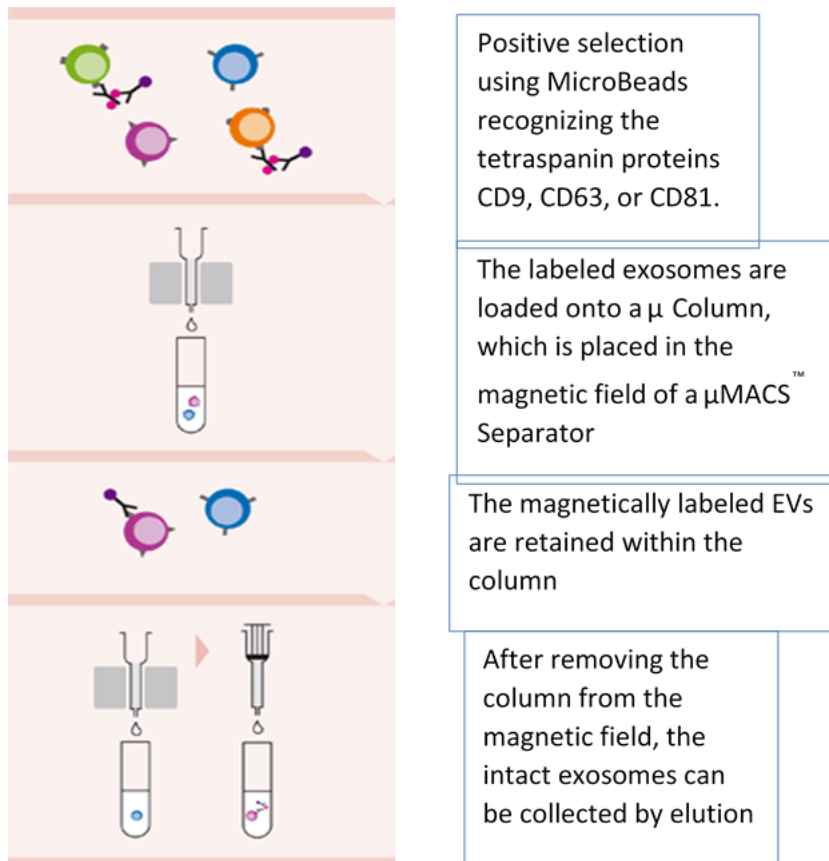
Figure 3. 1 Overview of Miltenyi Biotec Exosome Isolation Kit protocol

The second isolation method used was the ultracentrifugation (UC) technique, following incubation, exosome containing medium was removed from flasks and combined for centrifugation steps which were carried out at 4°C as outlined in Figure 3.2. The first two spins at 2000xg for 20 min and 10000xg for 30 min use standard centrifugation to remove dead cells and debris, the following spins at 100,000xg for 70 min use an ultracentrifuge which produces an exosome pellet which is washed with PBS and centrifuged, this pellet is resuspended in 25ml PBS and layered over 4ml of TRIS/Sucrose/D_2O solution and centrifuged at 100,000xg for 70 min, the sucrose layer was then removed using a 18G needle attached to a 5ml syringe and added to 25ml of PBS and a final spin of 100,000xg for 70min produces the purified exosome pellet which was resuspended in PBS and stored at -80°C.
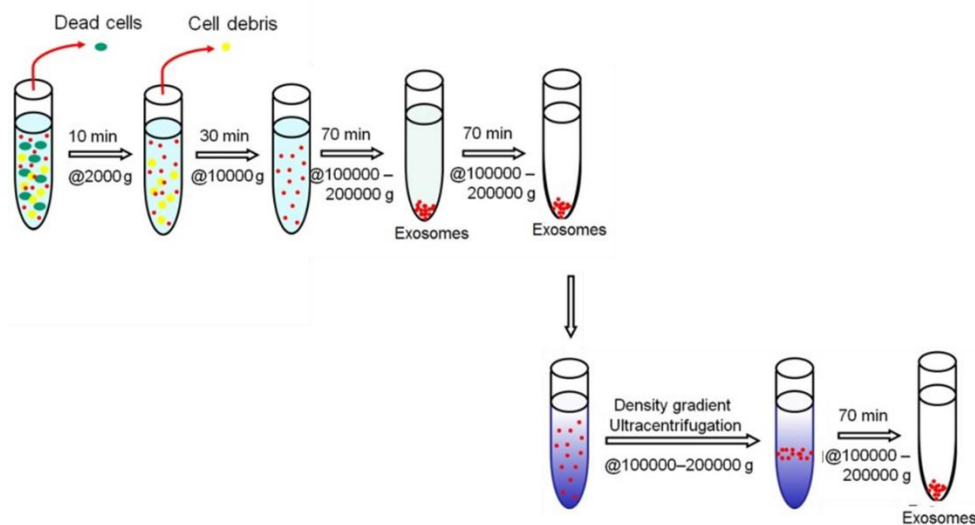
Figure 3. 2 Summary of exosome isolation using serial ultracentrifugation (adapted from (Li et al. 2017))

3.2.5 Confirmation of exosome content

Three methods were used to confirm exosome content according to the guidelines of the Society of Extracellular Vesicles (Thery et al. 2018), these included a Bradford protein assay to assess protein content, Nanoparticle Tracking Analysis (NTA) to assess particle content and western blotting to assess the presence of exosome protein markers.

1. Bradford protein assay

Method followed as outlined in Chapter 2 apart from the following alterations; exosome lysates were prepared by adding 10µl of exosome preparation to 25µl of cell lysis buffer. Exosome lysates were diluted 1 in 5 using 5µl and 20µl sterile water and aliquoted at 10µl per well in duplicate into 96-well plate before reading in spectrophotometer.

2. Nanoparticle tracking analysis (NTA)

NTA is a method that visualises and then quantitates nanoparticles (10-1000nM) in liquids. This laser-illuminated microscopical technique utilises the properties of light scattering and Brownian motion to analyse nanoparticles in real-time. NTA uses high-intensity laser beams sent through a sample chamber, the particles within suspension pass through the beam and cause a scattering of light which can be detected by a highly sensitive camera over multiple frames (Figure 3.3). NTA software 3.1 tracks

each particle and analyses the velocity of particle movement separately resulting in an estimate of particle size and distribution.

Exosome preparations were diluted using nanoparticle-free water (Fresenius Kabi, Runcorn, UK) so that the particle concentration (particles/ml) was within the linear range of the instrument (NanoSight NS300), (1:100-1:1000 dilution range). 100nM standard latex beads for calibration (Malvern Instruments) were prepared by diluting 1:1000 using particle free water, mixed and taken up into a 1ml syringe which was connected to NanoSight via rubber tubing. Before running exosome samples, tubing was washed with particle free water until no particles were detected on the screen. Diluted exosome preps were then administered and recorded under controlled flow using the NanoSight syringe pump (Malvern Instruments) set at 50µl/min and temperatures set at 25°C. Three replicate videos of 1 minute were taken and batch analysed using NTA 3.1 software with camera sensitivity and detection threshold set at 14-16 and 1-5 respectively using sCMOS Camera System (OrcaFlash 2.8, Hamamatsu C11440, Hamamatsu City, Japan), histograms for each triplicate measurement were generated and the area under each plot was averaged to be used as a particle concentration measurement and corrected for cell number.
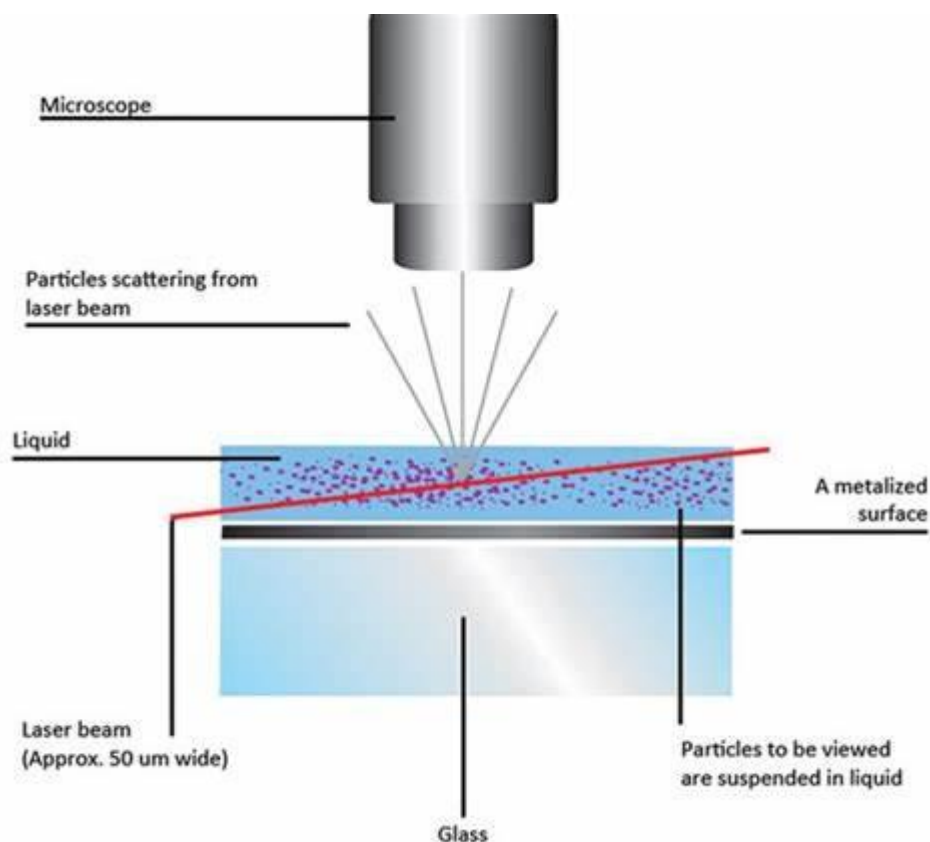
Figure 3. 3 Overview of NanoTracking Analysis instrumentation (www.photonics.com)

3. Western Blotting

Exosome lysates were generated and quantitated as outlined previously and western blotting was carried out as detailed in Chapter 2. 5-10µg of total protein from exosome preps and whole cell lysates were loaded per well and membranes were incubated with tetraspanin antibodies CD9, CD81 and CD63 and cytosolic antibody ALIX (Santa Cruz Biotechnology, Texas, U.S.A.) all used at 1:3000 dilution for exosome prep validation. Endoglin and CD73 used at 1:5000 dilution (Santa Cruz Biotechnology, Texas, U.S.A.) are mesenchymal stem cell stromal markers and GAPDH used at 1:1000 dilution (Santa Cruz Biotechnology, Texas, U.S.A.) which is used as a cytoplasmic originating marker for exosomes.

3.2.6 mIR preparation and identification

To investigate the miRNA content of exosome samples I used two commercially available kits to extract miRNA from supernatant collected from MSC cultures following five days incubation with exosome-free medium. The two kits are both manufactured by Qiagen, miRNeasy Serum/Plasma Advanced Kit (Cat. No. 217204) and ExoRNeasy Serum/Plasma Kit (Cat. No. 77044). Protocols were followed according to manufacturer's guidelines and resulting miRNA stored at -80°C.

To determine the integrity and quantify the miRNA yield from exosome preps I compared two methods; the Agilent 2100 Bioanalyzer Eukaryote total RNA 6000 Pico Kit (Agilent Technologies, Germany) which uses capillary electrophoresis to separate sample components, and detects them by fluorescence which translates into a gel like image and electropherogram for analysis. 1µl of miRNA sample was required and protocol was followed according to manufacturer's guidelines.

The second method used was the Qubit 2.0 Fluorometer (ThermoFisher Scientific, UK) which uses fluorescent dyes that upon binding to their target (specific to miRNA in this case) become intensely fluorescent, it then uses standards to quantitate. miRNA samples were diluted before running on instrument using the Qubit Working Solution at 1:200, if a sample was particularly weak the dilution was reduced to 3:200 dilution, protocol followed according to manufacturer's guidelines.

3.3 Results of exosome identification

3.3.1 Immunophenotyping

Exosome preparations were extracted from MSC cultures originating from normal bone marrow (NBM, n=7), AML diagnostic (n=10) and post-BMT early (1-2 months, n=4), mid (3-6 months, n=7) and late (9 months+, n=4). As outlined in Chapter.2 MSC cultures were immunophenotyped at stages of sub-culture to check the differentiation status and heterogeneity of stromal formation. When results were combined (Figure 3.4) there was a uniformity of stromal markers (CD73, CD90, CD105, CD166, CD44, CD146) present for MSCs from passage 2 with an associated loss of CD45 and other myeloid lineage markers (CD14, CD13, CD33, CD34).
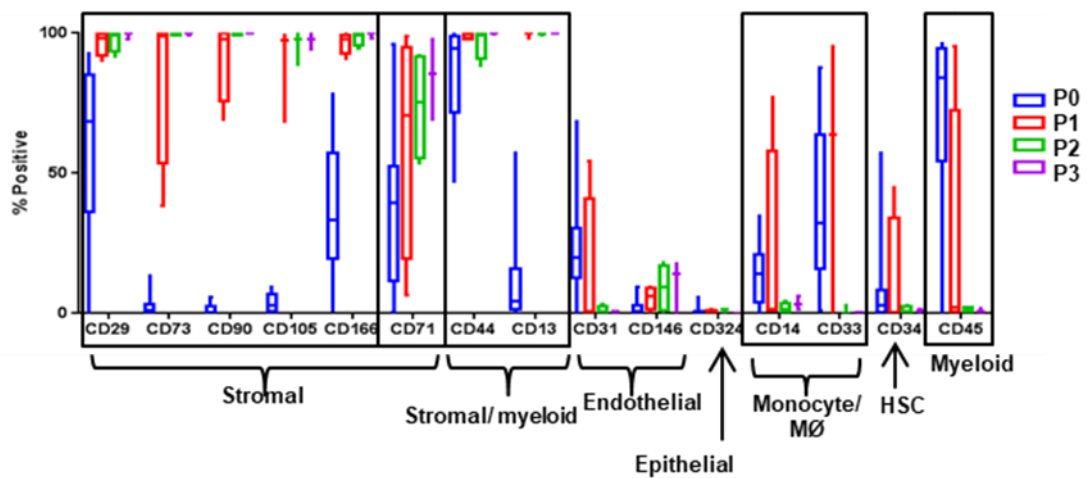
Figure 3. 4 Immunophenotypic flow analysis of primary AML cultures at various stages of sub-culture (P0 – P3), (n=32)

3.3.2 Protein quantification

The amount of exosome protein preparations per ml of MSC supernatant was significantly different between samples of different origin. Normal bone marrow and early BMT samples showed the lowest and smallest range of protein concentrations compared to diagnostic and mid-late post BMT which showed higher mean protein concentration and larger spread of values (Figure 3.5).
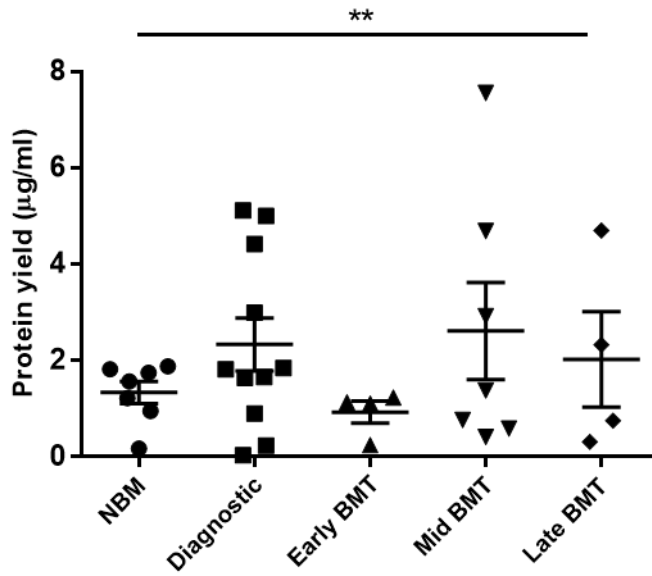
Figure 3. 5 Protein yield (μg/ml) of exosome preparations originating from MSC cultures of normal bone marrow (NBM), diagnostic AML and early (1-2 months), mid (3-6 months) and late (+6 months) post-bone marrow transplant (BMT) patients. (**p=0.007).

3.3.3 Western blotting

The presence of the transmembrane proteins CD81, CD63 and CD9 and cytosolic markers ALIX and GAPDH were used to identify exosomes within ultracentrifugation preparations, positive expression of these markers is shown in Figure 3.6 for exosome preparations produced from ultracentrifugation of normal (NBM), diagnostic (Diag) and post-BMT (BMT) MSC derived cells (A) and within the human stromal cell line HS5 (B).
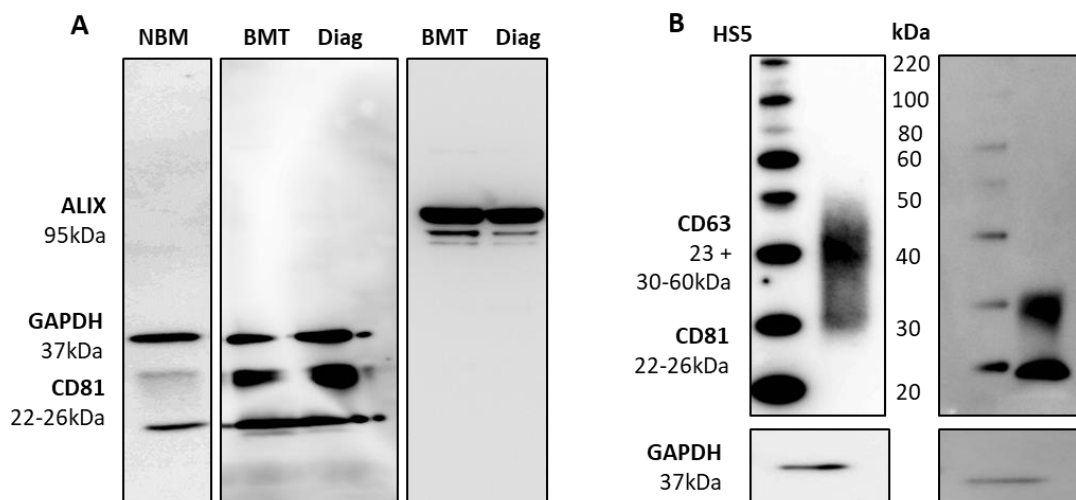
Figure 3. 6 Western blot showing the expression of exosome transmembrane markers CD81 and CD63 and cytosolic exosome markers ALIX and GAPDH within NBM, post-BMT and diagnostic derived MSCs (A) and HS5 cell (B) exosome preparations.

Western blots were run using whole cell lysate and the equivalent exosome preparations for HS5 cells and post-BMT AML derived MSCs (Figure 3.7). Within the HS5 cells the exosome associated marker CD81 and MSC associated stromal markers endoglin and CD73 were present within the exosome preparation but absent within the whole cell lysate (A) this was reflected in the post-BMT blot but using CD63 as the exosome associated marker (B), these results are an important measure of the purity of the exosome preparations produced.
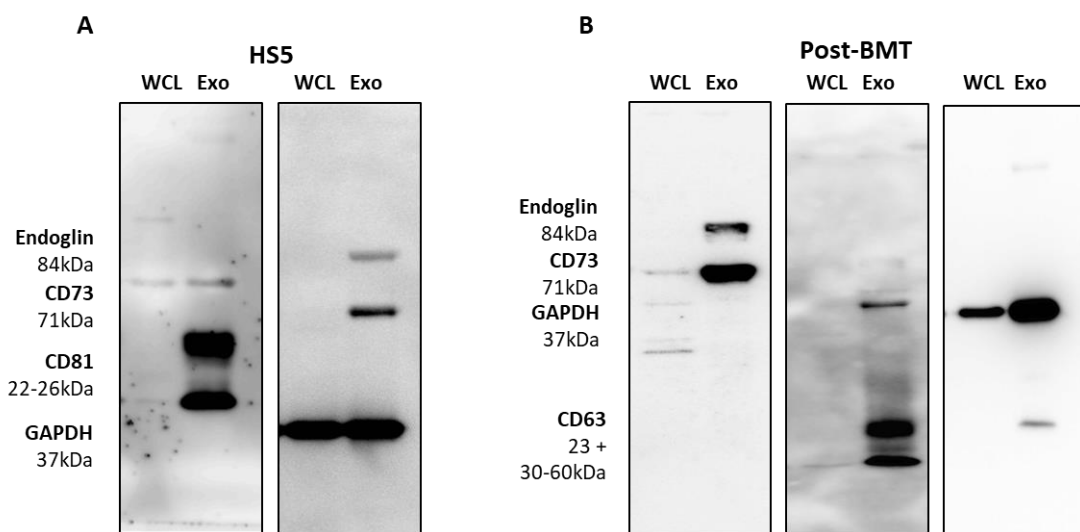
Figure 3. 7 Western blot comparing the expression of exosome markers (CD81 and CD63) and MSC stromal markers (CD73 and Endoglin) between whole cell lysate (WCL) and exosome preparations of HS5 cells (A) and post-BMT AML derived MSCs (B).

3.3.4   NanoSight

Electropherogram charts produced for each sample analysed by NanoSight provided useful information regarding quality of exosome preparation and the spread of EVs present. Introduction of the sucrose cushion to the ultracentrifuge protocol was tested using exosome preps from HS5 stromal cell lines and the sucrose addition provided a better quality of exosome preparation which although less concentrated showed an increased uniformity of microparticles <200nm highlighted by a smoother NanoSight histogram profile (Figure 3.8 A-B).
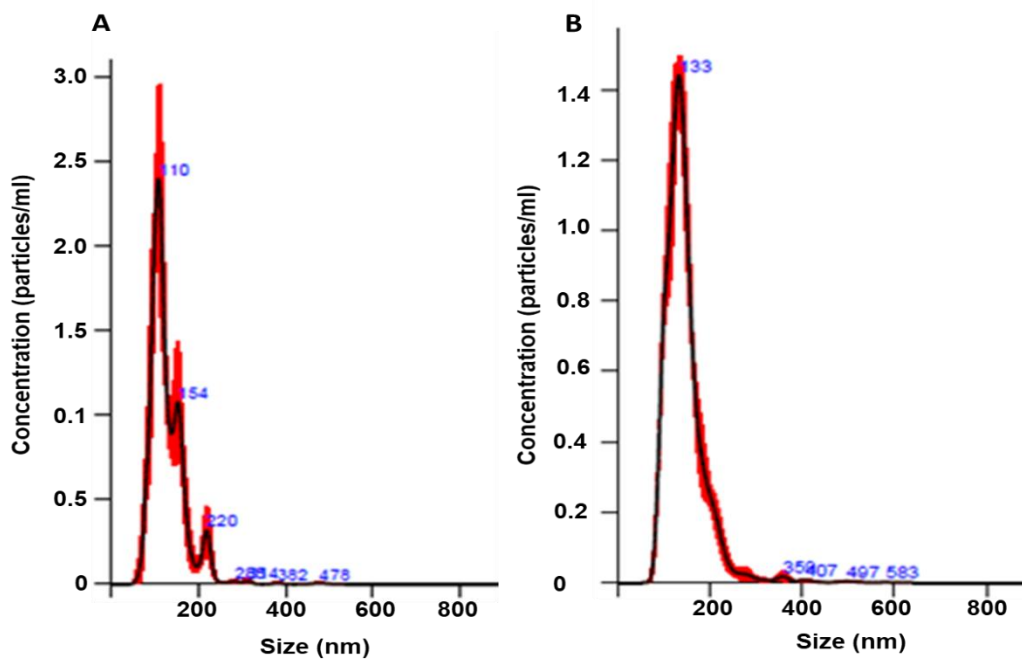
Figure 3. 8 Electropherograms from NanoSight analysis of HS5 derived exosome preparations using ultracentrifuge protocol without sucrose cushion purification step (A) and with (B).

Samples originating from NBM, diagnostic and post-BMT were analysed and post-BMT sub-categorised into early (1-2 months) and mid-late (3 months+). Comparison of nanoparticles per ml of harvested supernatant showed a significant difference between patient subtypes (Figure 3.9A). Mid-late post-BMT samples showed significantly higher number and range of nanoparticles (p=0.0066), minimal difference was observed between the other patient groups and particle distribution more uniform. Quantitation of the exosome preparation purity as dictated by the protein:particle ratio (protein concentration and NTA combined) showed a different configuration compared to the nanoparticle number assessment (Figure 3.9B). Diagnostic samples showed a higher number of protein:particle and a larger range compared to all other groups, NBM and mid-late BMT gave the lowest and more consistent values.

Protein:particle ratios were also assessed in serial MSC sub-cultures (Figure 3.9C) which was found to be relatively stable.
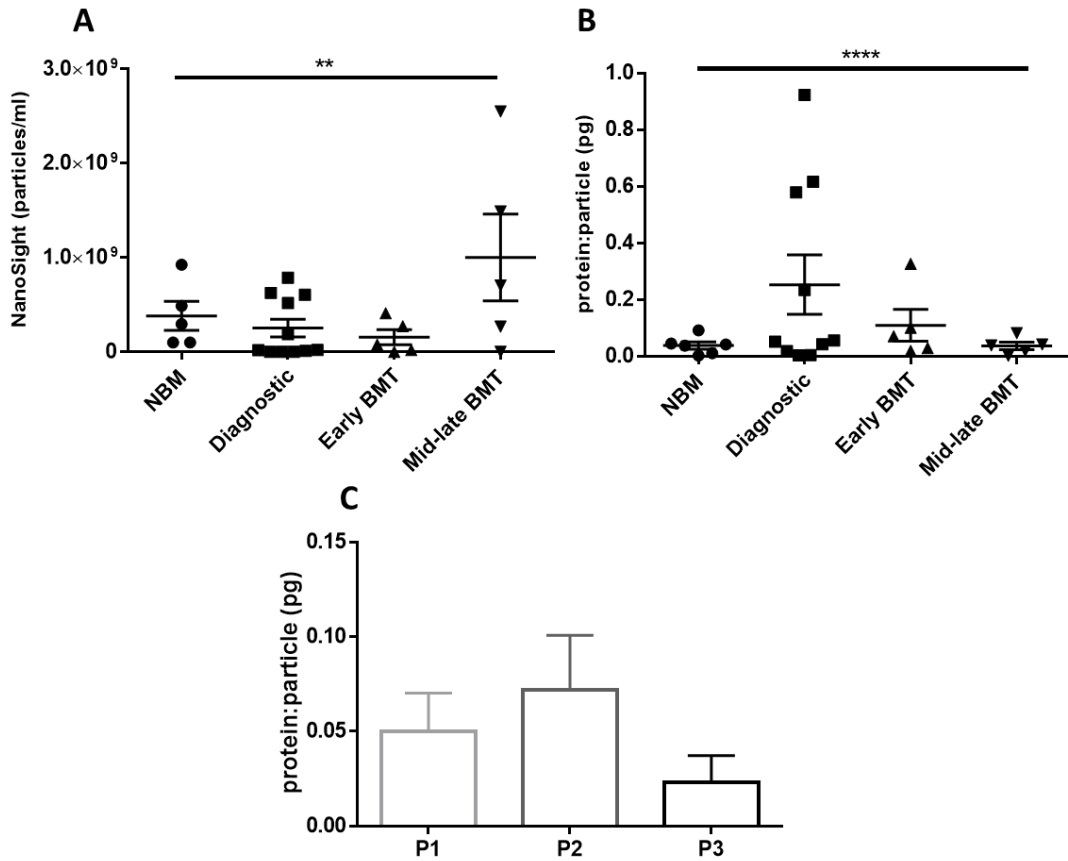
Figure 3. 9 (A) Chart showing NanoSight analysis of MSC derived exosome preparations originating from normal bone marrow (NBM), AML diagnostic, early post-BMT (1-2months) and mid-late post-BMT (>2months), particles/ml is the number of nanoparticles measured per ml of supernatant collected and extracted (**p = 0.0066). (B) Chart showing combination of exosome preparation total protein content with nanoparticle measurement as protein: particle ratio (pg) as a recognised measure of exosome sample purity (****p = <0.0001). (C) Chart showing the effect of sub-culturing MSCs on their exosome production (n=10).

NanoSight analysis gave the size of each nanoparticle measured within the exosome preparations assessed, which allows sub-categorization of particle content for each sample into <100nM, 100-200nM and >200nM. The relative amount of each category was then calculated as a proportion of total particles to assess the range of EVs present in each sample (Figure 3.10)
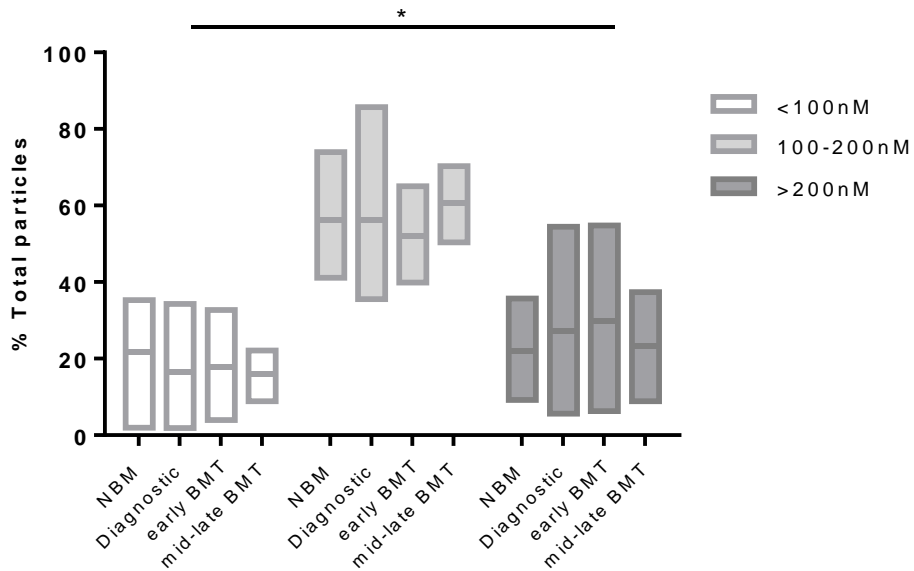
Figure 3. 10 Chart comparing the presence of nanoparticles within the category <100nM, 100-200nM and >200nM of exosome preparations derived from patient sub-groups as measured by NTA using NanoSight instrument. (*p = <0.0001, n = 28).

3.3.5 Microscopic Analysis

Average measurements from multiple fields of view of MSC cultures were combined to give a total cell count per field of view and an average cell area calculated from each flask. In order to compare samples, I used the size of the flask of origin and number of flasks cultured to calculate an average cell number which could then be cross-referenced with the exosome production of that MSC culture. Figure 3.11 shows examples of pictures acquired of MSC cultures from different patient origins.
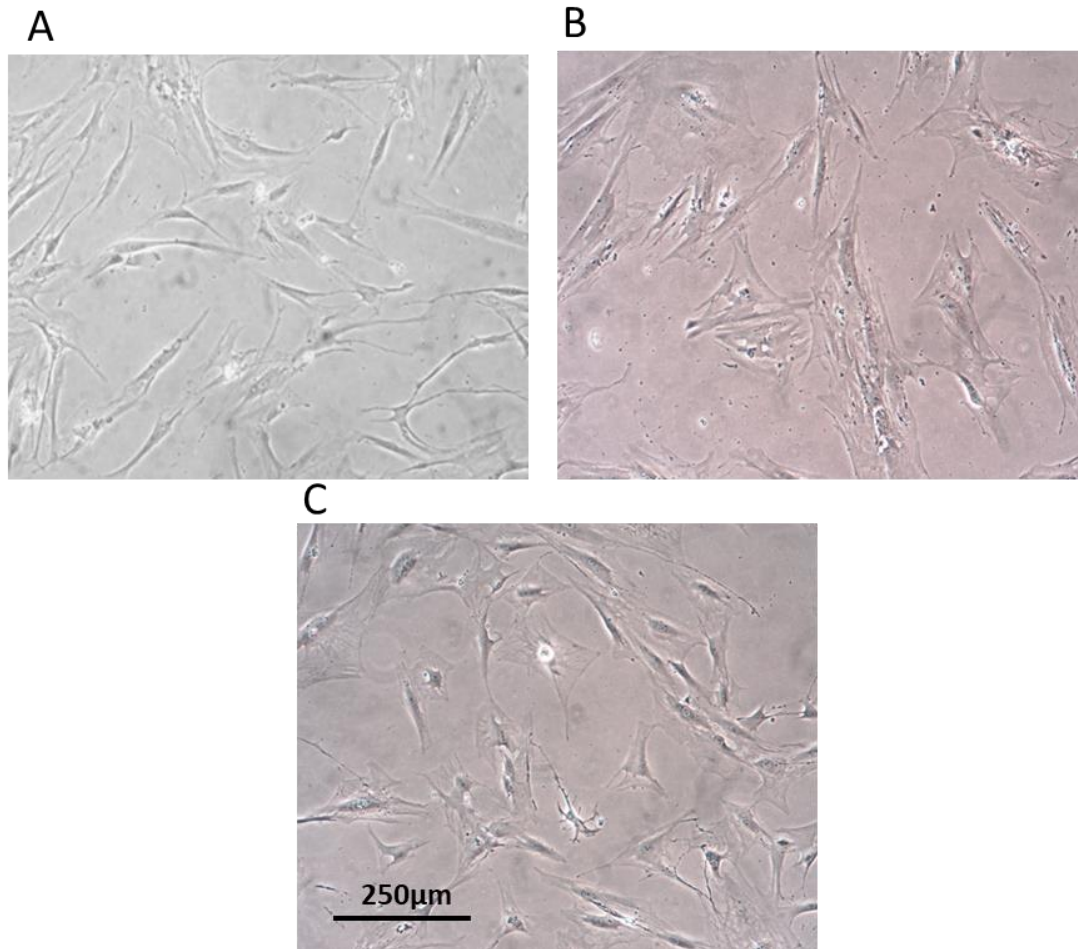
Figure 3. 11 Pictures of MSC cultures from cells of NBM (A), AML diagnostic (B) and AML post-BMT origin (C). (Pictures taken with Nikon Microscope 10x magnification and GXCapture-T software)

The number of exosomes released per MSC was significantly different between disease stage groups, with post-BMT derived MSCs associated with the highest and largest range of exosome numbers (Figure 3.12A). NBM derived MSCs showed the lowest exosome numbers. Cell size varied significantly, diagnostic MSCs were largest, NBM smallest and post-BMT showed largest range in size (Figure 3.12B).
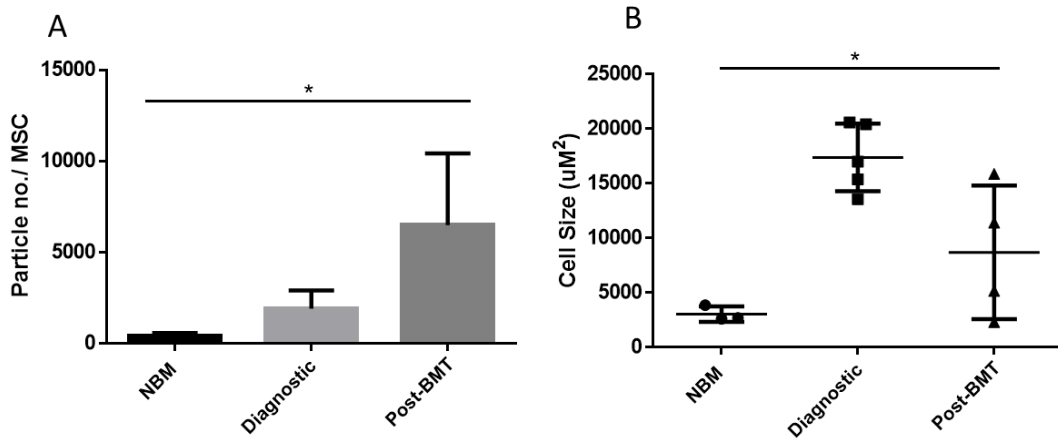
Figure 3. 12 (A) Chart showing number of exosomes produced per MSC derived from NBM, diagnostic and post-BMT AML derived MSC cultures (*p=0.0374, n = 12). (B) Chart showing microscopic cell measurement of MSC cultures (*p=0.0151).

In order to fully characterize the exosome preparations, I combined microscopic analysis of cell size and cell number with exosome protein loading and number of exosomes produced per cell for corresponding samples. The results showed minimal associations, number of cells vs particle loading showed no correlation (Figure 3.13C) and cell size showed no impact on exosomes produced per MSC (Figure 3.13A). A trend could be observed within the particle loading vs cell size, the highest particle loading was produced by cultures containing larger cells originating from diagnostic source (Figure 3.13B).
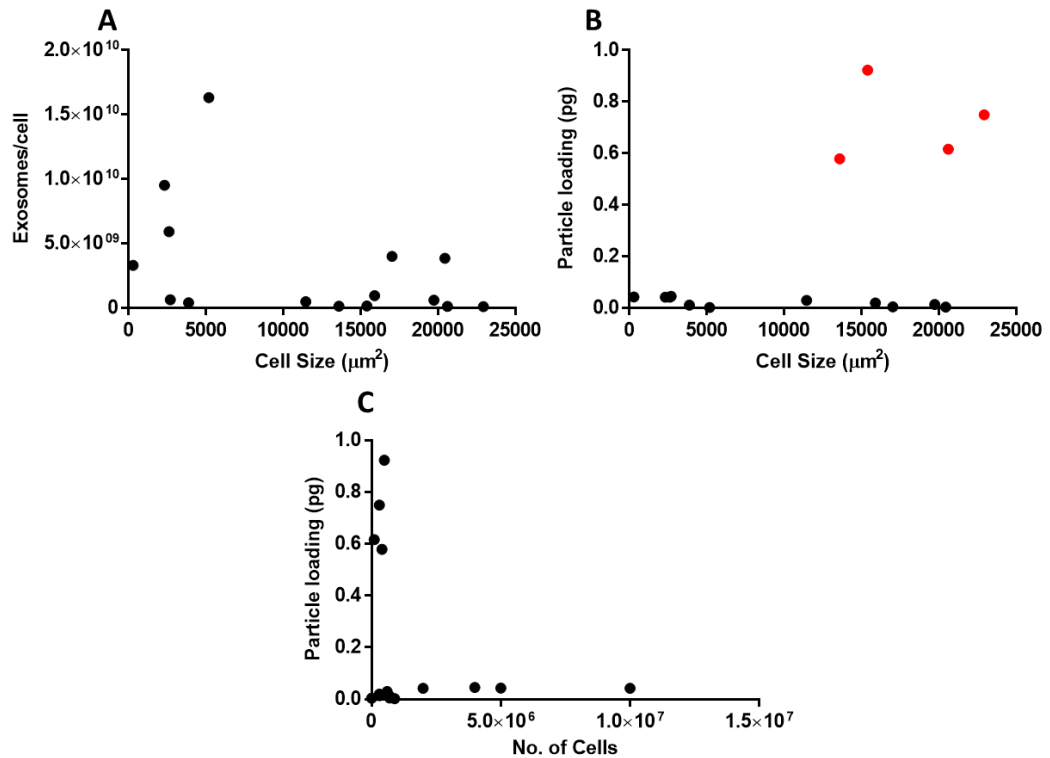
Figure 3. 13 Charts showing the association of MSC cell size with exosomes produced per cell (A) Amount of protein loaded into each exosome (particle purity) the points in red denote diagnostic patients (B) and number of MSCs in culture with exosome particle protein loading (C).

3.4   Results of exosome content

3.4.1   mIR quantification

Exosome fractions were assessed for mIR quantity using the Agilent Bioanalyzer to analyse miRNA produced by Qiagen miRNeasy and ExoRneasy Kits. Samples were run on the Eukaryote total RNA 6000 Pico chip. Total RNA control samples were run alongside a group of miRNA preps produced from both kits. Results of the gel image show two clear bands for the control RNA (Figure 3.14A), which were absent from all other samples as would be expected for cell-free derived mIR sample, these bands correspond to ribosomal RNA which in humans 28s has ~5070 nucleotides and 18s has 1869 nucleotides and is commonly expressed as a ratio of 28s/18s to indicate that purified RNA is intact and hasn't degraded. The electropherogram showed a clearer picture (Figure 3.14B-C), the control total RNA has two clear 18s and 28s peaks as expected, this is not the case within the miRNA samples which show the highest concentration of RNA below 30bp showing that the RNA being analysed is too small to pick up by this instrument and give an accurate measurement.

As the Agilent Bioanalyser samples contain high levels of small RNA (Garcia-Elias et al. 2017), it lacks the sensitivity needed to accurately quantitate these very small molecules from cell-free systems such as exosomes or plasma secretory mIR samples and RIN assessments of sample quality are unusable. Therefore Qubit analysis was undertaken as a more accurate and sensitive way to quantify miRNA samples.
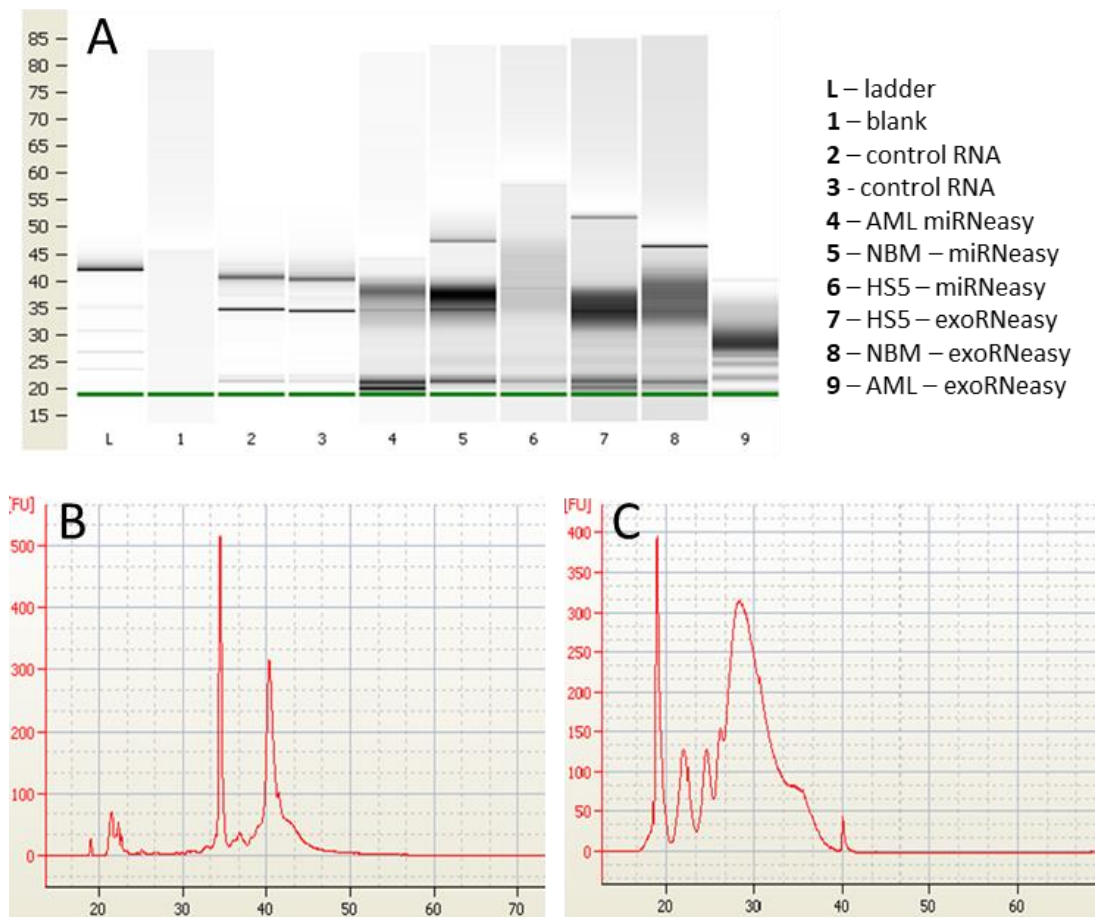
Figure 3. 14 Agilent Bioanalyzer results for miRNA analysis. Gel like image (A) showing control RNA and miRNA extracted from miRNeasy and ExoRNeasy kits. Electropherogram comparing control total RNA (B) with miRNA originating from HS5 cells (C).

Following extraction of miRNA and quantitation I compared results from the ExoRNeasy Kit (Figure 3.15A) and RNeasy Kit (Figure 3.15B), for both kits the diagnostic samples produced the highest yield and greatest variability of miRNA. ExoRneasy kit harvested higher concentrations of miRNA for all sample types, this agrees with latest research which recommends Qiagen ExoRneasy as the best overall kit for better quality miRNA prep as shown by the Qubit analysis, although RIN number absent from this measurement as it is not appropriate within a cell free sample (Laurent 2018).
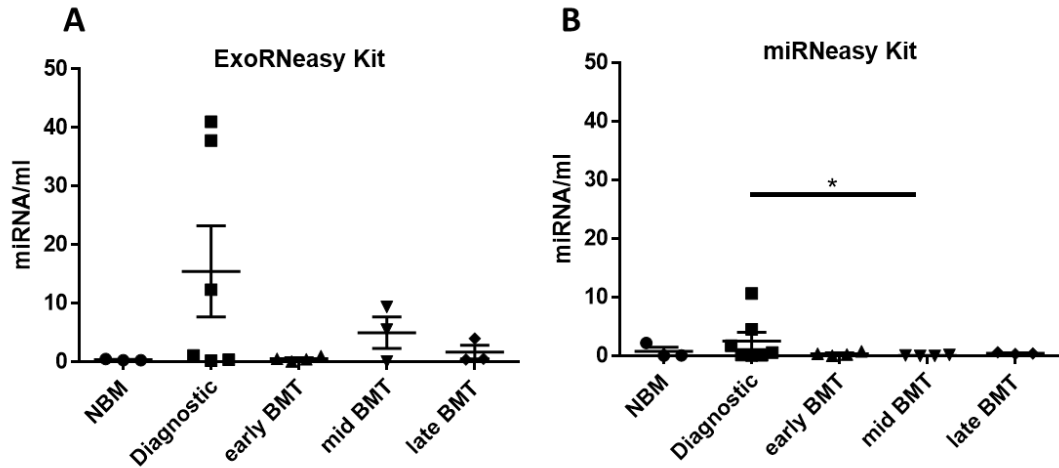
Figure 3. 15 Charts showing miRNA harvest from MSC cultures of NBM, AML diagnostic and post-BMT origin using Qiagen ExoRneasy Kit (A) and Qiagen miRNeasy Kit (B) (*p=0.0424).

Correlations between miRNA harvested from both Qiagen kits and cell size of the associated sample were investigated (Figure 3.16A-B) and a trend for miRNA yield to increase with cell size was observed.
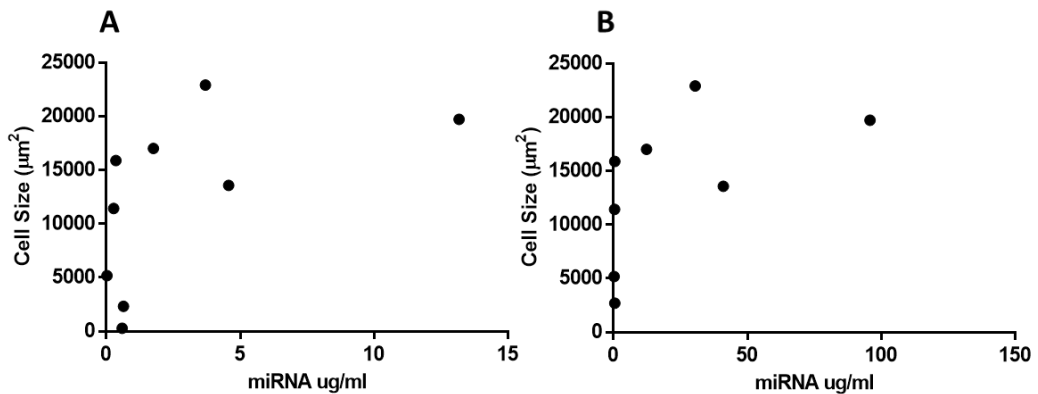
Figure 3. 16 Charts expressing correlations between exosome cell size and miRNA yield from ExoRneasy Kit (A) and RNeasy Kit (B).

3.5 Discussion

After comparing two standard protocols for exosome isolation I decided to use the ultracentrifugation technique for isolation of exosomes to be used in downstream functional and molecular analysis. Ultracentrifugation is the most common method of extracting exosomes in published research and is recommended as a relatively easy technique with little technical expertise needed and minimal long-term costs and most importantly a pure preparation of exosomes.

Although ultracentrifugation is a very long and labour intensive technique compared to the Miltenyi Biotec Exosome Isolation Kit, it is undoubtedly the superior isolation method. The Miltenyi Biotec Kit although quick and easy produced exosomes that were irreversibly attached to Microbeads which meant that I was unable to carry out quantitative analysis using NTA, as the beads interfered with the laser beam tracking device. The beads also interfered with running of electrophoresis gels for immunoblotting analysis and could not be used in functional assays, limiting their use solely to molecular characterisation.

The addition of a sucrose cushion purification step within the ultracentrifugation protocol added more stages to the technique but results of a comparison of exosome preparations produced from HS5 cells (Figure 3.8), clearly showed that this additional purification step produces an exosome extract that contained a more uniform type of vesicle within the exosome defined limits. A sucrose cushion is not always necessary as stated in the Society of Microvesicle Guidelines (Thery et al. 2018) the level of purity of your exosome prep should be influenced by your downstream requirements. I found that the material lost during the sucrose cushion stages of the protocol is substantial, significantly lowering exosome protein yield, this causes problems when attempting to run a western blot as it is very difficult to obtain enough exosome protein in order to successfully visualise on a nitrocellulose membrane. In the case of western blotting, I found that removing the sucrose cushion purification step and lysing the exosome pellet directly was more effective when this form of analysis was needed for validation of exosome presence rather than content. In the case of functional assays the extra purification step is necessary in order to ensure the exosome preparation added to cell culture assays is as pure as possible.

The phenotyping of MSCs as they were sub-cultured showed that past passage 2 cells had lost their myeloid lineage characteristics and obtained a uniform stromal associated phenotype across all samples (Figure 3.5). When looking at the effect of early passaging on exosome production no significant change in particle number or protein amounts was observed (Figure 3.9C).

Analysis of the exosome protein and nanoparticle content showed for both there were significant differences between the MSC clinical sub-categories (Figure 3.5, 3.9A). Samples of diagnostic origin showed highest exosome protein content along with mid-late BMT, both groups showed large range of values. Nanoparticle Tracking Analysis (NTA) showed high particle number and greater spread of values in mid-late BMT. Combination of both forms of measurement in the form of protein: particle ratio

showed diagnostic samples produce highest number of exosomes and gave largest spread of data. Previous studies have shown high numbers of exosome release from AML cells compared to normal, confirmed by my findings (Szczepanski et al. 2011) but this is the first case of post-transplant assessment in AML.

It is interesting to observe the exosome production differences between post-BMT, mid-late is higher than early BMT and shows similarity to those levels seen in NBM. It is not completely unexpected to see similar results for early-BMT and NBM as leukaemic burden is reduced/absent after conditioning and donor engrafted cells return the marrow to a phenotype similar to that of a normal individual. The increase seen in exosome production at later stages of post-transplant could be due to a variety of possibilities. Exosomes are important modulators of immune response especially in cancer development they can both potentiate an immune response or act as an immunosuppressive (Ruivo et al. 2017). Following transplant a patient will wean off immunosuppressants and re-build their natural immune defences, this could signify an increase in exosome secretion from T-cells which would promote development of immune response and help regulate the formation of synapse between T-cells and antigen-presenting cells (Barros et al. 2018). Some studies have shown a beneficial impact of exosomes on engraftment. An *in vivo* study involving heart allograft MSC derived exosomes were added post-transplant resulting in the induction of Tregs and reduction of CD8+ T-cells, reducing allograft immune responses and inducing immunotolerance (He et al. 2018).This was also reflected in a study involving the addition of exosomes derived from Treg cells which resulted in the prolonged survival and function of kidney allografts (Yu et al. 2013).

In contrast, exosomes could also act in a negative manner within the post-transplant environment, as studies have shown that increased exosome quantity released into circulation along with their associated cargo could help increase the expression of certain proteins which promote T-cell mediated GvHD (Vallabhajosyula et al. 2017) ,(Fleissner et al. 2012).The increase in diversity of exosome levels in later stages of post-transplant could represent the heterogeneous response to the treatment that patients display and may represent immune reconstitution or potentially GvHD and GvL response. Further characterisation of patient status and outcome may be available for individual samples and will be requested, although much larger cohorts of material would be required to confirm this observation.

Cellular stress is another factor that could bring about an increase in exosome release as cells readjust to the changing microenvironment. Previous studies have shown

that post-transplant, cell stress can stimulate the release of exosomes from donor cells that express mismatched HLA and a lung allograft targeted immune response triggered (Ravichandran et al. 2019).

The other potential implications of increased exosome level observed in mid-late transplant samples is the indication of MRD and potential disease resurgence. It has already been suggested that exosomes derived from AML could be used to detect MRD although this has not yet been put into practice, as confirmation of this process is currently constrained by lack of reliable techniques for isolation of exosomes from different origin (Boyiadzis M 2016).

Exosomes isolated from breast cancer patients were shown to express the marker protein CD24 but not EpCAM protein, this differed from those exosomes isolated from normal exosomes that expressed both and so could be used as a biomarker for prognosis or diagnosis (Rupp et al. 2011). It has also been determined that EDIL3 and fibronectin are present on extracellular vesicles of breast cancer patients and levels reduced in concordance with surgical treatment enabling them to be used as indicators of therapeutic response (Moon et al. 2016a; Moon et al. 2016b).

NTA analysis using NanoSight measures the size of all particles for each sample analysed which enabled me to sub-categorise each sample into % composition of the following groups; <200nM (small microvesicles), 100-200nM (exosomes) and >200nM (large microvesicles) (Figure 3.10). Between categories for all samples there were significantly more particles of size 100-200nM, confirming that all sample preparations were composed of exosomes in the highest quantity. There was no significant difference seen between the clinical groupings although once again the largest range in particle size within the 100-200nM group was within the diagnostic samples, this agrees with all previous observations and the large spread of data perhaps is linked to the characteristic heterogeneity of AML.

Microscopic analysis of MSC cultures highlighted a significant difference between clinical categories when comparing number of exosomes produced per cell as calculated using microscope cell counts and NTA (Figure 3.12A). Cells producing the lowest number of exosomes originated from NBM as expected (Hong et al. 2014a) and diagnostic and post-BMT showed higher exosome number and spread of data.

Cell size analysis (Figure 3.12B) showed that the smallest cells were observed from NBM, diagnostic cultures contained the largest cells and a range of cell sizes were seen in post-BMT cultures. This observation agrees with previous work that showed an overall increase in cancer cell size compared to normal. The heterogeneity of the

post-transplant cell size could reflect the different stages of bone marrow recovery experienced within this cohort of patients (Shashni et al. 2018).

miRNA was extracted using miRNeasy and ExoRNeasy Kits. Agilent Bioanalyzer was initially used to quantitate the miRNA but was rejected due to problems quantifying small RNA, giving inaccurate measurements of miRNA concentration. I successfully quantitated using Qubit Instrument which due to it's increased sensitivity could accurately measure miRNA levels in all samples. ExoRneasy produced higher yield of miRNA from the same samples as RNeasy and as other studies have also concluded, is the best choice (Garcia-Elias et al. 2017).

miRNA produced from both kits produced a higher yield from diagnostic samples (Figure 3.15A-B) suggesting an association between high levels of miRNA and increased numbers of exosomes in these samples. Previous work agrees with this and has shown that amount and composition of exosomal miRNA differs between patients with disease and healthy individuals (Zhang et al. 2015).

In summary:

- Ultracentrifugation best choice for exosome isolation only use sucrose cushion purification step if carrying out functional downstream applications

- ExoRNeasy Kit is best for miRNA extraction and Qubit instrument most sensitive quantitation.

- Diagnostic samples show highest exosome production and miRNA content which agrees with previously published work

- No correlation found with MSC size and exosome production, but rather production activity is related to disease stage.

- Increased miRNA yield shows some association with higher exosome production as seen in diagnostic samples

- Early-BMT exosome production shows a stabilization of exosome production similar to that of NBM.

- Mid-late BMT exosome production shows a much larger spread of responses in which some retain NBM like levels and others return towards their diagnostic levels which could be in response to cell stress or potential indicator of MRD/relapse.

4 Chapter 4: Validation and Functional Exosome Assays

4.1 Introduction

4.1.1 Exosome function within the bone marrow microenvironment

Many studies have investigated the function of cancer cell derived exosomes and exosome interaction within the tumour microenvironment is a growing field exploring the major role in intercellular communication between cell types helping to support tumour growth, differentiation, proliferation, angiogenesis, immune evasion and drug resistance.

It has been reported that exosomes help block cell differentiation and modulate target cells via miRNA transfer. Previous work has shown that exosomes originating from endothelial and haematopoietic cells disrupt Notch signalling through miR126 transfer resulting in an increase of cell differentiation (Liao et al. 2018). Exosomes derived from other cell types including neuronal progenitor cells and macrophages, have also been shown to promote differentiation usually from naïve to mature cell type. This process is not restricted to healthy cells, exosomes released from malignant cells have been shown to block their own differentiation and inhibit the differentiation of non-supportive cells (Yu et al. 2007).

Exosomes within the malignant microenvironment serve as good candidates for biomarkers of disease. They contain genetic and proteomic contents that can reflect the cell of origin and protect their cargo from potential degradation from nucleases and proteases. This increases the biomarker half-life and protects the sample integrity for successful downstream analysis. This has successfully been used to monitor disease in a wide variety of cancers such as lung, breast and colon(Corcoran et al. 2011; Cazzoli et al. 2013; Ogata-Kawata et al. 2014). Studies in AML have shown that exosome levels present in patient plasma reflect disease load, and after a course of induction chemotherapy levels of exosome protein significantly reduce concomitant with that of leukaemic blasts in the bone marrow. Other work has highlighted the presence of TGF-β1 levels in exosomes derived from AML patients following chemotherapy, where exosomal TGF-β1 levels were significantly reduced in patients with long-term complete remission, suggesting that changes in exosome derived TGF-β1 levels may reflect therapeutic response (Hong et al. 2014b).

Exosomes have been shown to modulate angiogenesis by stimulation or inhibition, these effects are dependent on exosome content and surface molecule expression

factors, which are normally under the direction of the stimulus which induces exosome release. Mechanisms involved in the stimulation of exosome angiogenesis include the transfer of mIRs e.g. MIR-126, proteins e.g. CKIT and lipids e.g. S1P90 along with activation of various signalling pathways. One of the most common being ERK1/2 and the transfer of transcription factors. Exosome directed inhibition of angiogenesis, is known to take place through the mechanisms of LDL receptor mediated endocytosis and induction of oxidative stress.

Pancreatic cancer exosomes have been shown to induce the fibrotic migration inhibitory factor and TGFβ leading to microenvironment remodelling creating favourable conditions for liver metastasis (Costa-Silva et al. 2015). Studies have shown another factor which further enhances the role of exosomes in pre-metastatic niche formation is the presence of PD-L1 on their cell surface. A study on metastatic melanoma showed that when this binds to PD-1 found on macrophages, T and B-cells, it creates an immunosuppressed microenvironment which increases disease progression (Chen et al. 2018).

Studies have investigated the function of cancer derived exosomes and immune response. MHC+ exosomes from both tumour and immune cells have been shown to bring about a T-cell specific immune response (Andre et al. 2002). In contrast other studies have reported the opposite effect of exosomes, within a malignant environment aiding immune surveillance evasion and activating macrophage inflammatory responses (Abusamra et al. 2005; Yu et al. 2007).

Studies on leukaemia derived exosomes have shown that exosomes released from leukaemic cells alter their proliferative and migratory response. AML derived exosomes have been shown to transfer mRNA to surrounding bone marrow stromal cells, a study involving human derived exosomes enriched with CXCR4 and IGF-IR transcripts were co-cultured with murine bone marrow stromal target cells and showed the factors were present in the recipient cells, confirming the functional consequences of AML exosome trafficking (Huan et al. 2013). Within the bone marrow microenvironment, CML derived exosomes were shown to stimulate the release of IL8 from the surrounding stromal cells, helping to promote disease survival (Corrado et al. 2014). Studies have also shown that CML derived exosomes transfer mIR-126 directly to endothelial cells, targeting CXCL12 and VCAM1 mRNA increasing the adhesive and migratory capacity of the CML cells (Taverna et al (Taverna et al. 2014).

Studies involving multiple myeloma (MM) derived exosomes showed that when co-cultured with MM cells a transfer of mIR-15a took place from MM-derived exosomes which resulted in increased MM cell proliferation and survival. This was absent from normal bone marrow-MSC derived exosomes which instead showed growth inhibition of MM cells (Roccaro et al. 2013).

An undesirable function of exosomes within the therapeutic environment is their ability to horizontally transfer drug resistance, as seen in a study using MM cells co-cultured with bone marrow stromal cell derived exosomes, as well as increasing proliferation of the MM cells they also induced drug resistance to Botezomib (Wang et al. 2014), studies suggest that this is thought to be due to the intercellular transfer of miRNA by exosomes from drug-resistant to sensitive cancer cells (Litwinska et al. 2019).

Several studies have investigated the potential benefits of using combinations of novel drugs to overcome AML chemo-resistance, but when combination therapies are tested in stromal co-culture a much higher therapeutic dose is required suggesting stromal niche contributes to resistance (Weisberg et al. 2008). Further co-culture studies using primitive leukaemic populations showed that as well as displaying drug resistance these cells are non-cycling and retain their leukaemic genetic mutations such as FLT3-ITD (Alvares et al. 2011; Parmar et al. 2011).

This highlights the important function exosomes play in providing a form of communication between cancer and host, aiding disease progression.

4.1.2   miRNA profiling in exosomes

miRNA can be transported as cargo within exosomes, or extracellular miRNA can be loaded onto high density lipoproteins or bound by Argonaute2 protein on the outside of the exosome. miRNA cargo is thought to be well protected via all modes of exosome transport (Arroyo et al. 2011; Tabet et al. 2014).

Once exosome miRNA is delivered to the recipient cell it plays a functional role, which consists of typical negative regulation that confers changes in expression levels of specific target genes involved in many biological processes such as apoptosis, proliferation, and immune response. Recently, novel functions of exosome mediated mIR transfer have been identified, where exosome miRNA acts as a ligand binding to toll-like receptors and activating immune cells (Fabbri et al. 2012).

Large scale microarray studies of primary cells and cell lines originating from melanoma, breast and ovarian cancer, show a high frequency of genomic alterations within miRNA loci, suggesting that the abnormal miRNA expression in malignant cells

could arise from amplification or deletion of specific genomic regions containing miRNA genes (Zhang et al. 2006).

Studies have shown that miRNA is also susceptible to epigenetic modulation. In AML mIR-223 expression was epigenetically silenced by the AML1/ATO fusion protein through CpG methylation (Fazi et al. 2007).

4.1.3 Stromal support in AML

As previously summarized, MSCs of the bone marrow microenvironment have been recognised as playing a major role in leukaemic progression, supporting AML growth and mediating chemoresistance. It has been shown *in vitro* that co-culture with AML patient stromal cells, drives resistance to Ara-C in some patient samples and these patients are associated with a poor clinical outcome. This resistance is mediated by both direct cell to cell contact and also the soluble secretory molecules released from the bone marrow stromal cells, that protect AML cells from Ara-C induced cytotoxicity (Macanas-Pirard P 2017   ).

The effect of exosome release from both AML and stromal cells have previously been found to incite leukaemic behaviour, in the form of increased cell proliferation, immune evasion and drug resistance. In one study, normal and AML derived stromal exosomes conferred a resistance to Ara-C drug treatment, however only AML-derived exosomes inhibited the efficacy of the drug AC220, suggesting a differential therapeutic response between NBM and AML stromal cells (Viola et al. 2016).

A recent study used reverse protein array technology to look at key proteins involved in a number of signalling pathways associated with stroma-mediated AML survival within both primary and cell line AMLs, and showed that the stroma reprogrammed a number of signalling networks, including PI3K/AKT/MTOR and BCL2 which modified the sensitivity to several targeted agents (Zeng et al. 2017).

Similarly, profiling of exosomal protein content is a relatively new field of research, and a recent study of plasma exosomes in epithelial ovarian cancer identified 294 proteins within both cancerous and non-cancerous samples, the results of which could be used for diagnostic and prognostic purposes (Zhang et al. 2019). It has also been suggested that MSC derived exosomes work through a protein based mechanism of action and that proteins could be the main driver of therapeutic action as opposed to miRNA which is found at significantly lower levels compared to protein in exosomes (Toh et al. 2018).

Given the clear evidence of stromal protection and modifying effects on AML blasts during chemoresistance and residual disease, further characterisation of exosome mediated signalling pathways, and functional phenotypes involved is required to assess their contribution to stromal support.

4.2 Methods

4.2.1 Luminex Assay

In order to investigate the content of primary AML MSC derived exosome and matched supernatant samples I used a Luminex Assay as a way of profiling disease specific biomarkers. Luminex is a multiplex immunoassay which can simultaneously detect and quantify multiple target analytes within complex sample types and only requires small volumes of sample, an overview of the technique is shown in Figure 4.1.
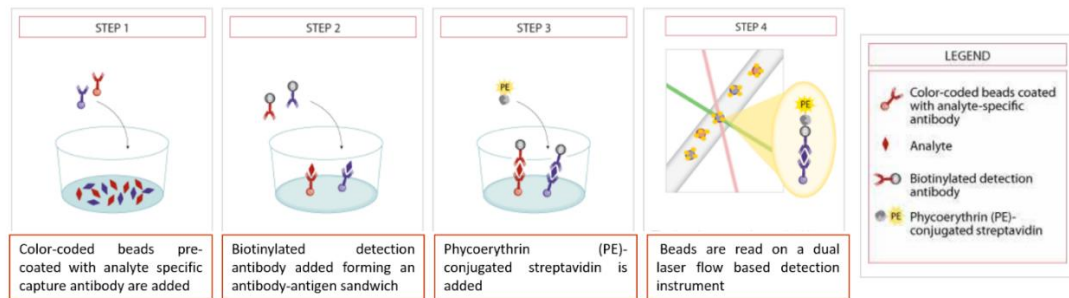
Figure 4. 1 Summary of Luminex assay principle (www.rndsystems.com)

Exosome preparations were lysed using a cell extraction buffer as outlined in Chapter.2. The lysis buffer is important as it needs to contain enough detergent to solubilise the proteins within the sample but avoid such high concentrations that will interfere with assay sensitivity, for example using buffers containing ionic detergents such as traditionally used RIPA buffer. The buffer we chose to use contained low levels of ionic detergent SDS which we diluted 1:10 before running to ensure correct final concentration and no downstream interference.

Custom made Human Premixed Multi-Analyte Kits for luminex analysis (R&D Systems Inc, Minneapolis, U.S.A) were designed to encompass a panel of 104 cytokine/survival targets detailed in Table 4.1.

44-plex	38-plex	20-plex
Adiponectin	BMP-2	April
B7-H1/PD-L1	BMP-4	BAFF/BLyS
CCL2/MCP-1	BMP-9	CCL5/RANTES
CCL20/MIP-3 alpha	C5/C5a	CXCL9/MIG
CCL24/Eotaxin-2	CCL22/MDC	ErbB2/Her
CCL4/MIP-1 beta	CCL3/MIP1 alpha	FABP4/A-FABP
CD25/IL-2 R alpha	CD117/c-kit	Fibroblast Activation Protein
CD30	CEA/CEACAM-5	Galectin-3
CD31/PECAM-1	CEACAM-1/CD66a	Gas6
CD40	CXCL1/GRO alpha	IGFBP-3
CD44	E-Selectin	IGFBP-4
CXCL1/GRO alpha	EGF	IL-1ra
CXCL10/IP-10	EphA2	Lipocalin-2/NGAL
CXCL11/ITAC-1	ErbB2/Her2	MMP-1
CXCL12/SDF-1 alpha	Fas Ligand	MMP-3
CXCL13/BLC/BCA-1	FLT3 Ligand	MMP-9
CXCL2/GRO beta	HGF	N-Cadherin
CXCL4/PF4	ICAM1	RBP4
CXCL5/ENA-78	IL-12 p70	TGF-alpha
CXCL8/IL-8	IL10	TRAIL
Dkk-1	Insulin	
Fas	Leptin	
FGF acidic	LIF	
G-CSF	MCAM/CD146	
GM-CSF	MIF	
IFN-gamma	NCAM1-1/CD56	
IL-1 alpha	Osteopontin	
IL-1 beta	P-Selectin	
IL-10	Protein S/PROS1	
IL-13	S100A8	
IL-17A	SCF	
IL-2	Serpin E1/PAI-1	
IL-22	SPARC/Osteonectin	
IL-27	TGF alpha	
IL-3	TIMP-1	
IL-31	TPO	
IL-4	VCAM-1	
IL-5	VEGF-A	
IL-6		
IL-7		
L-Selectin		
Resistin		
TNF RII		
TNF-alpha		

Table 4. 1 Luminex multiplex panel targets

Luminex Assay was run as outlined in Chapter.2.

4.2.2 Taqman Multiplex Assay

miRNA was extracted using Qiagen ExoRNeasy Kit as previously described and samples were diluted accordingly using sterile water and used at a concentration of 1µg of miRNA per reaction. TaqMan Advanced miRNA and cDNA Synthesis Assay Kits (ThermoFisher Scientific, Massachusetts, U.S.A) were used and manufacturers protocols followed to carry out poly(A) tailing, adaptor ligation, reverse transcriptase and mIR-Amp reactions. All reactions were run on GeneAmp PCR System 9700 V.3.10 (Applied Biosystems Ltd.). The mIR-Amp reaction product was diluted 1:10 with 0.1X TE buffer (Fisher Scientific UK Ltd) and 5µl used per reaction using TaqMan Advanced miRNA Assay Kit for targets mIR155, mIR1246 and housekeeping control mIR361. Reactions were set up in MicroAmp Optical 96-well plates (Applied Biosystems Ltd.) in triplicate including a water blank and positive control and run on QuantStudio 5 System (ThermoFisher Scientific, Massachusetts, U.S.A.) using fast cycling mode and programme as manufacturer's instructions. Data was analysed using software package QuantStudio Design and Analysis Desktop Software Version 1.4.3 which generated Ct values for each sample which I then used to calculate 2$\triangle\triangle$Ct to assess relative expression levels for each target.

4.2.3 Tissue Culture Functional Assays

4.2.3.1 Cell Proliferation Assay

HS5 cells were maintained and seeded onto 48-well tissue culture plates in advance as described in Chapter 2. MV411, KG-1a, HS5 and primary AML cells were cultured (as described in Chapter 2) and seeded +/- HS5 stromal layer as required at $7.5x10^5$ cells/ml primary AML cells and $1x10^5$ cells/ml cell lines. Exosome preparations (generated through sucrose cushion density gradient) were made up to the required concentration using sterile 1XPBS before adding directly to culture and placing into 37°C incubator for 48 hours. Following incubation supernatant was removed and cells pelleted for flow cytometry as outlined in Chapter.2. In order to assess cell viability and suspension vs adherent fractions flow cytometry was carried out using CD45 to identify stromal and myeloid cell populations.

4.2.3.2 Migration Assay

HS5 cells were seeded into 24 well chemotaxis tissue culture plate (Corning Costar #3421) as described in Chapter.2. 24 hours before start of assay plate inserts were rinsed with sterile 1x PBS and pre-warmed medium added to receiver wells.100µl of chemotaxis medium was added to inserts and transferred to a well containing medium +/-HS5. KG-1a cells were counted and 100µl added at $2x10^5$/ml into the plate inserts.

Exosome preparations were isolated by sucrose density gradient and made up in sterile 1XPBS to add at 200µg per receiver well. Inserts containing cells were added back into plate and incubated for 24 hours. To harvest, the inserts were removed and contents transferred to centrifuge tube, insert was rinsed with 250µl of medium and combined, this procedure was repeated with the wells and HS5 stromal layers trypsinised and harvested as described in Chapter 2. All cell extracts were centrifuged at 1200rpm and flow cytometry carried out as described in Chapter.2 with CD45 and 7AAD for cell viability assessment.

4.2.3.3   Cell Glo drug cytotoxicity assay

Pacritinib (molecular weight 472.58) was supplied by CTI Biopharma Corporation (Seattle, WA, U.S.A) in 0.5% methylcellulose (w/v) and 0.1% Tween-80 in H2O, made up to working stock concentration of 10mM by dissolving in DMSO and diluted in IMDM media to a top dose of 100 µM. 50µl of IMDM was placed in each well of an opaque walled 96-well plate, excluding the outside wells where evaporation is more likely to occur. Top dose wells were made up to 100ul with further medium and drug added from the top dose Pacritinib to give a 2x of final concentration. Serial dilution using 50µl from the top wells was applied across the 96 well plate resulting in 10 doses in triplicate. Control wells were set up using DMSO at equivalent concentrations to that found in the top dose wells. Finally, 50µl of primary AML cells +/- exosomes diluted in IMDM from 5×10^6/ml suspension were added to all the drug and DMSO wells (8×10^4/well) and then plates were incubated at 37°C, 5% CO2 for 48 hours. Cell Titer Glo (Promega) assay was performed to determine the number of viable cells that remained; this is a luminescent viability-based assay where the number of viable cells in a culture can be determined based on their ATP content, which signals the presence of metabolically active cells. At the end of 48 hours, Cell Glo reagent was defrosted at room temperature and shielded from light. Plates were removed from the incubator and allowed to equilibrate to room temperature for 1 hour (to prevent temperature gradients which may affect the rate of the luciferase reaction), then 100µl Cell Glo was added to each well and the plates were then shaken protected from light on an orbital shaker for 2 minutes to allow complete lysis of cells. Plates were read using a Chameleon automated plate reader (Hidex, Finland) on single direct luminescence setting with the aperture adjusted for RLU in the range of 20,000 – 5,000,000. The amount of ATP and therefore the luminescence reading is directly proportional to the number of live cells present in culture within this range. Dose response curves were established for cell viability as a percentage of the untreated/DMSO control and software package Calcusyn version 2.1 (Biosoft,

Cambridge. UK) was used to calculate a 50% inhibitory concentration (EC_{50}) for cytotoxic responses to Pacritinib +/- exosomes based on the luminescence data.

4.2.3.4   Western blotting – ERK

KG1a cells were cultured +/- HS5 cells as previously described in triplicate T75 tissue culture flasks and exosome free medium added overnight to HS5 cells. Medium was removed the following day and replaced with KG1a cells in exosome free medium at $2x10^5$cells/ml. MEK-PD (Sigma-Aldrich PZ0162) resuspended in DMSO was added to relevant flasks at 10µM and 100µM and DMSO alone added to control flasks for 48 hours. Exosome supernatants were removed and centrifuged at 1200rpm prior to exosome extraction and protein lysis carried out as described in Chapter.2. KG1a cell pellets were lysed for western blot analysis as previously described. Western blotting was carried out as previously described in Chapter 2 and the following antibodies were used to probe: ERK (Cell Signalling 4370) at 1:5000 dilution, CD81 (Santa Cruz SC-166029) at 1:1000 dilution and GAPDH (Santa Cruz SC-47724) at 1:5000 dilution. Rabbit secondary antibody was used at 1:25000 dilution.

4.2.3.5   Western blotting – Luminex Validation

Western blotting was carried out as previously described in Chapter.2 using exosome lysates and probed using Her/erbb3, ICAM-1, GAPDH and CD81 at dilutions as described in Table 2.1.

4.3   Results

4.3.1   Luminex Target Identification

Supernatants and matching exosome preparations derived from MSC cultures of normal, AML diagnostic, early post-BMT and mid-late post-BMT origin were collected and as described previously prepared for luminex anlysis. Samples were analysed on a custom panel of 104 targets and following analysis were quality checked to remove any failed targets or samples.
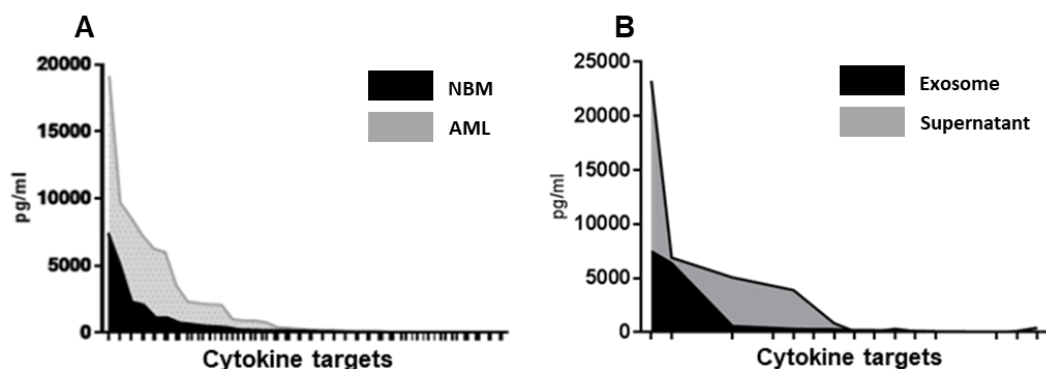
Figure 4. 2 Chart comparing average detection levels of cytokine targets as analysed by Luminex comparing NBM to AML samples (A) and exosome to supernatant samples (B) (n=18).

Targets were ranked in order of abundance and normalised to total protein load. AML derived stromal exosomes contained higher levels of targets overall compared to NBM derived stromal exosomes (Figure 4.2A). When compared to matched supernatant secretory profiles, exosome profiles contained fewer detectable targets than the parental secretory supernatant although some targets showed equivalent levels of abundance to that of the supernatant (Figure 4.2B).

To look at the distribution of data profiles Partek Genomics Suite 5 was used to identify clustering patterns. Figure 4.3 shows a hierarchichal cluster chart for exosome (A) and supernatant (B) samples. Exosome samples show a clear division between high overall expression of targets in NBM and diagnostic samples compared to post-BMT samples. Further investigation into these clusters identified Leptin, osteopontin, LIF, C-kit, insulin, BMP4, ccl22, TIMP-1 and serpin-e1 as the targets more highly expressed in NBM and diagnostic compared to post-BMT samples which showed low expression.

Supernatant samples showed a trend for NBM and diagnostic separating out into clusters. Diagnostic samples showed higher levels of ICAM-1, LIF, MIF, CXCL9, BAFF, APRIL, CXCL1, EphA2 and Fas ligand. In NBM higher levels of MMP-3, CCL5, IL-1RA and CEACAM-1.
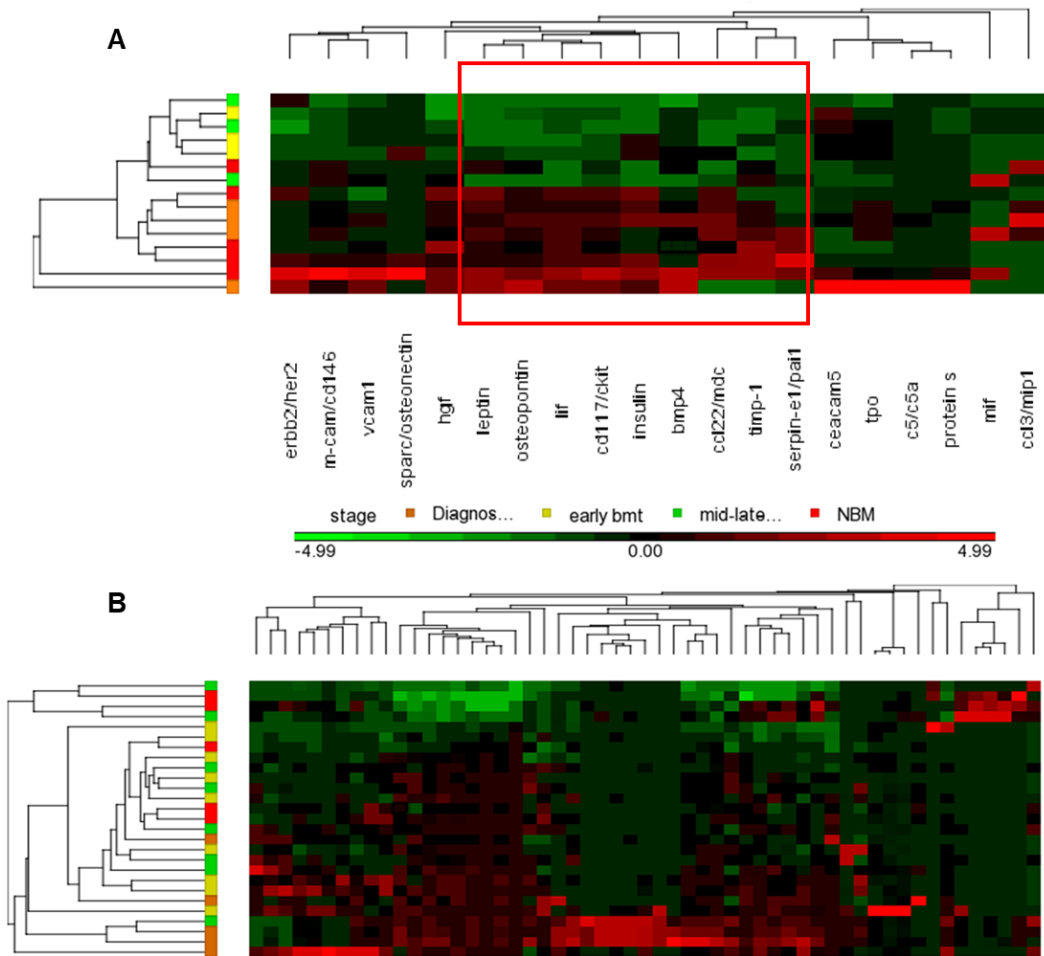
Figure 4. 3 Hierarchichal clustering analysis of NBM, diagnostic AML, early AML post-BMT and mid-late BMT MSC derived fractions of exosome (n=20) (A) and supernatnant (n=37) (B)

Prinicpal component analysis (PCA) is a statistical method which uses an algorithm to convert a set of variables which could be correlated, into a set of values that are linearly uncorrelated, it then attempts to explain the maximum amount of variance with the fewest number of principal components. A chart showing a representation of PCA analysis of exosome sample luminex content (Figure.4.4A) shows disctinct clustering of each disease stage with NBM samples show the largest spread of data, and post-BMT showing least variability. Early and mid-late BMT samples showed the most similar common profiles and diagnostic samples show the least amount of correlation to post-BMT samples.

PCA analysis of supernatant samples (Figure.4.4B) show the largest spread of data within the diagnostic samples, early post-BMT show the least amount of data variability and NBM and mid-late BMT show most correlation to each other.
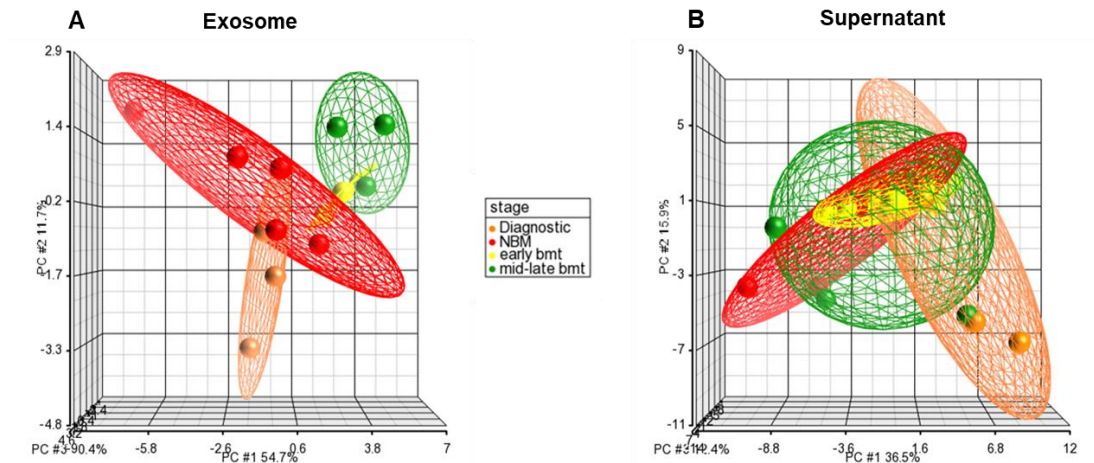
Figure 4. 4 PCA (principal component analysis) of NBM, diagnostic AML, early AML post-BMT and mid-late BMT MSC derived fractions of exosome (n=20) (A) and supernatnant (n=37) (B)

In order to further investigate target differences between patient groups and cytokine expression, exosome luminex targets were sub-categorised according to their function; immunology, survival/differentiation, adhesion and chemoattract and their overall abundance plotted for each sample group (Figure 4.5). VCAM-1 was the target most highly expressed within all sample types. ICAM-1 was also expressed highly within NBM, diagnostic and mid-late BMT. IL-4 and IL-10 were most highly expressed in early BMT and IGFBP-3 in mid-late post-BMT.
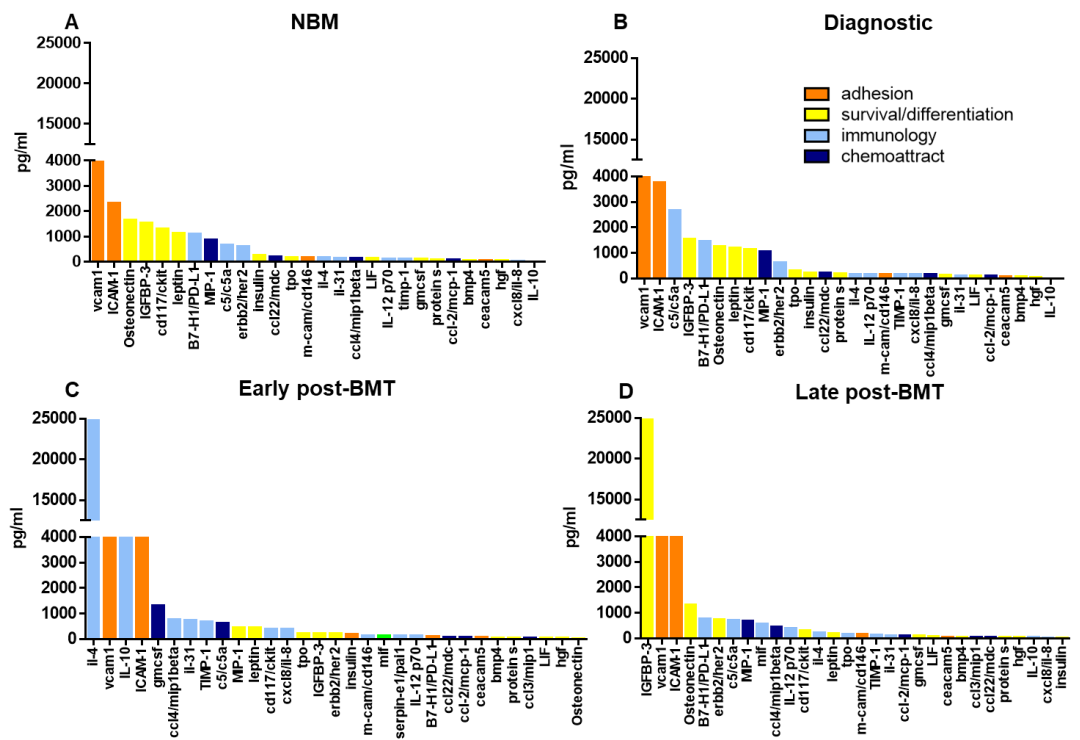
Figure 4. 5 Summary of Luminex target abundance levels from exosome samples derived from NBM (A), Diagnostic (B), early post-BMT (C) and mid-late post-BMT (D). Targets are categorised according to function; adhesion, immunology, chemoattract and survival/differentiation (n = 20).

Results show that there are differences between patient sub-sets and target abundance levels between exosome and supernatant samples. The pie charts in Figure 4.6 summarise the sample type and levels of expression of particular functional targets present within exosome and supernatant samples to try to identify more clearly potential changes. It is clear that NBM and diagnostic samples differ considerably between exosome and supernatant but become more similar within post-BMT. Both sets of sample show a big change in sample makeup post-transplant, exosome samples show highest levels of adhesion related targets, this changes early post-BMT to a predominatly immunological profile and then survival/differentiation late post-BMT.

Supernatant fractions show profiles at NBM and diagnostic which are heavily survival/differentiation biased, this changes early post-BMT to a predominantly immunology based profile as seen in exosomes and mid-late post BMT the profile reverts back to survival/differentiation.

In summary, within both exosome and supernatant NBM and diagnostic show similar content, this changes post-BMT exosome content differing to supernatant but both showing a change with time. This is useful information as it highlights the importance of serial timepoint sampling in order to achieve accurate monitoring of disease progression.

90
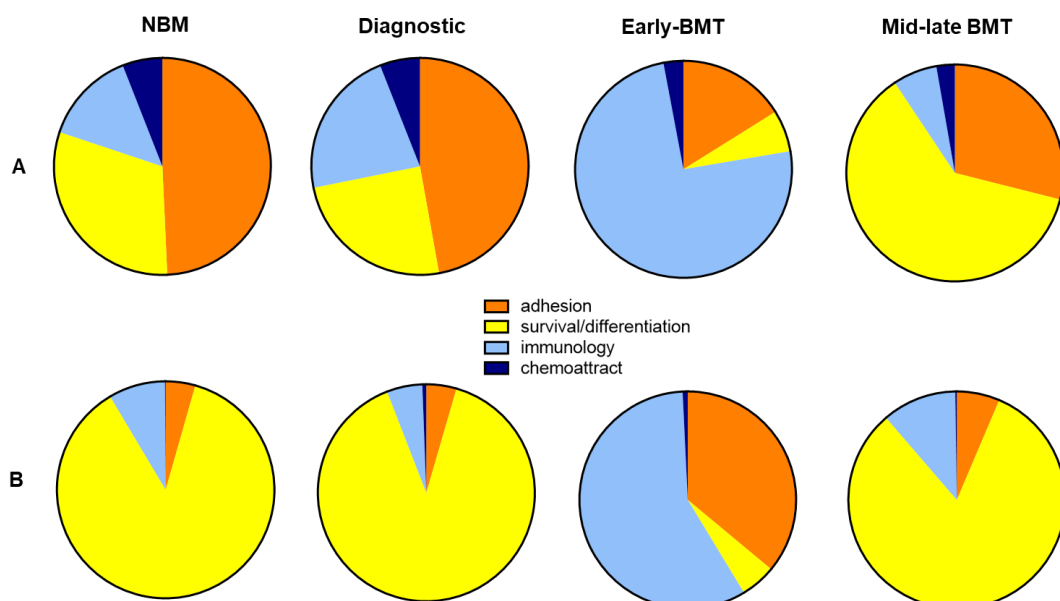
Figure 4. 6 Pie Chart showing proportional representation of targets associated with adhesion, survival/differentiation, immunology and chemoattract present within exosome (A) and supernatant (B) samples derived from MSC cultures of NBM, AML diagnostic and AML post-BMT (n = 20).

In order to further explore the highlighted differences seen between patient treatment stage for individual targets I plotted them individually for exosome and the accompanying supernatant abundance levels for comparison in order to identify similar trends of target abundance between the two sample types (Figure 4.7-4.10).

Within the immunology panel of exosome samples (Figure 4.7) a trend of increasing target expression levels from NBM and diagnostic through to post-BMT was observed although this was only significant for Erbb2 (p=0.0404). This trend was not reflected within the supernatant samples.

Figure 4. 7 Chart showing levels of immunology targets in normal, AML diagnostic and AML post-BMT MSC derived exosome (n=20) and supernatant (n=37) fractions as measured by Luminex immunoassay (* p=0.0404).

Survival/differentiation targets (Figure 4.8A-B) of exosome preps showed consistently lower levels within post-BMT samples for all targets except IGFBP-3 and showed a significant difference of; HGF (p=0.0357), BMP-4 (p=0.0286), protein-S (p=0.0159) and c-kit (p=0.0159). LIF also showed a significantly higher level of expression in diagnostic compared to NBM (p=0.0485).

This pattern of expression was not repeated in the supernatant fractions, only IGFBP-3 showed significantly lower levels of expression in post-BMT (p=0.0120), expression levels were elevated in diagnostic samples but not to a significant extent, in general the greatest variation was seen in the supernatant compared to the exosome samples.

A

B
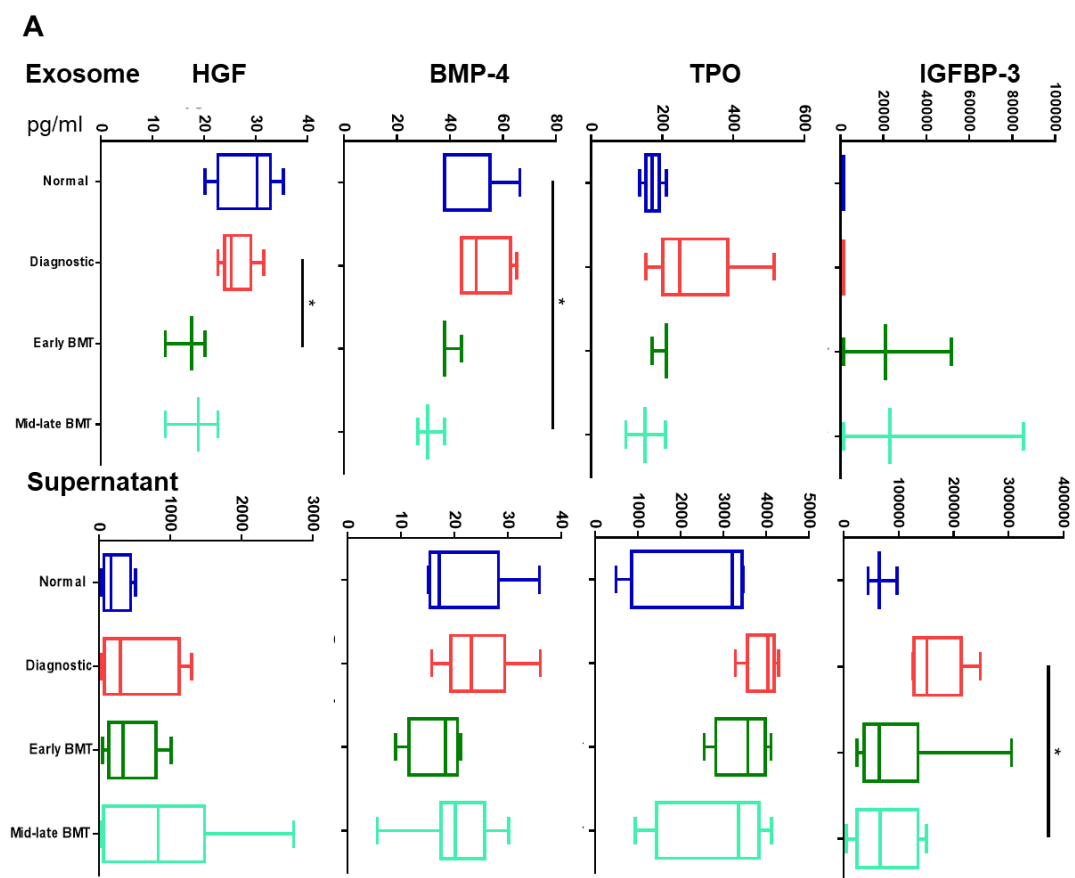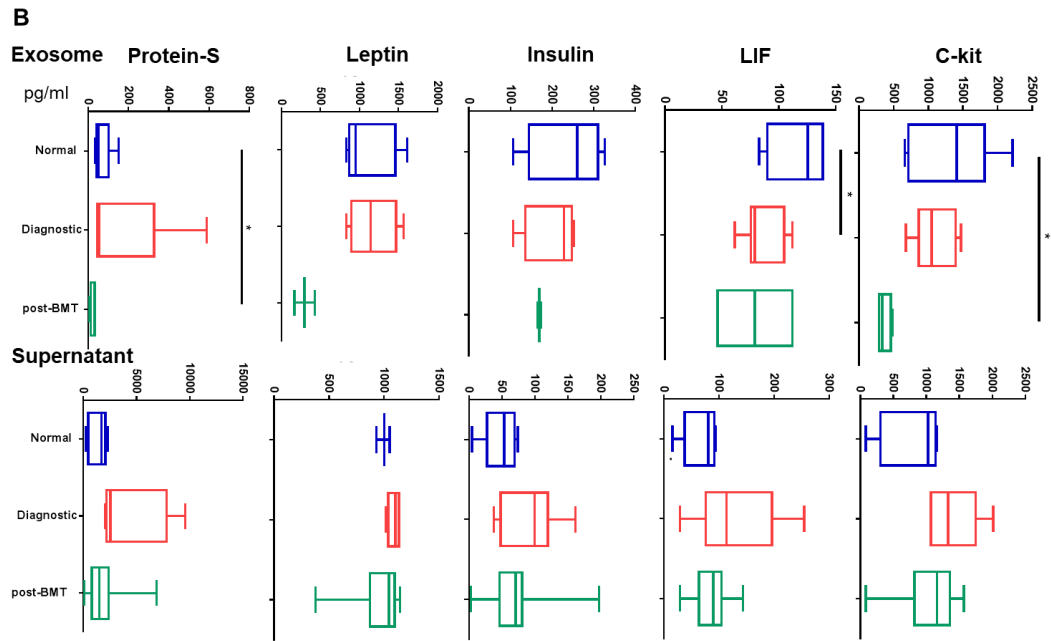
Figure 4. 8A-B Chart showing levels of survival/differentiation targets in normal, AML diagnostic and AML post-BMT MSC derived exosome (n=20) and supernatant (n=37) fractions as measured by Luminex immunoassay. *p= <0.05

Adhesion related targets (Figure 4.9) show higher levels of expression for NBM samples for VCAM-1 and significantly MCAM (p = 0.0357) in exosome fractions which is lost in post-BMT samples. Supernatants also show higher levels of expression for these targets and significantly for MCAM (p = 0.0349) within the diagnostic samples.
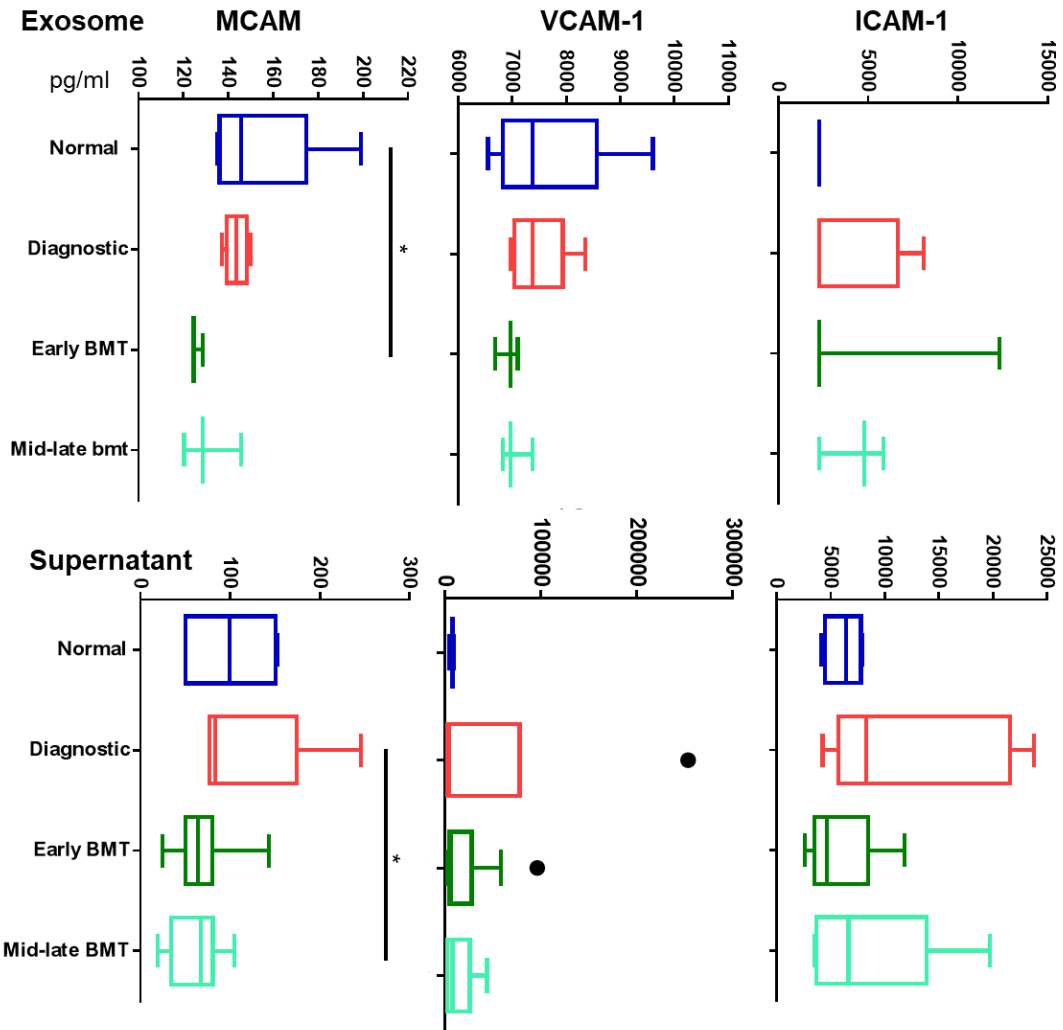
Figure 4. 9 Chart showing levels of adhesion targets in normal, AML diagnostic and AML post-BMT MSC derived exosome (n=20) and supernatant (n=37) fractions as measured by Luminex immunoassay. *p = <0.05

Chemoattract targets in exosomes don't show any significant difference between any of the patient sub-categorys for exosomes but they do show a trend towards higher levels within post-BMT samples for CCL2 and CCL4. This trend is reflected in the supernatant fraction for CCL22 which is significantly more highly expressed in post-BMT (n=0.0238). In CCL2 and CCL4 the levels of expression are highest within the diagnostic samples but this is not significant.
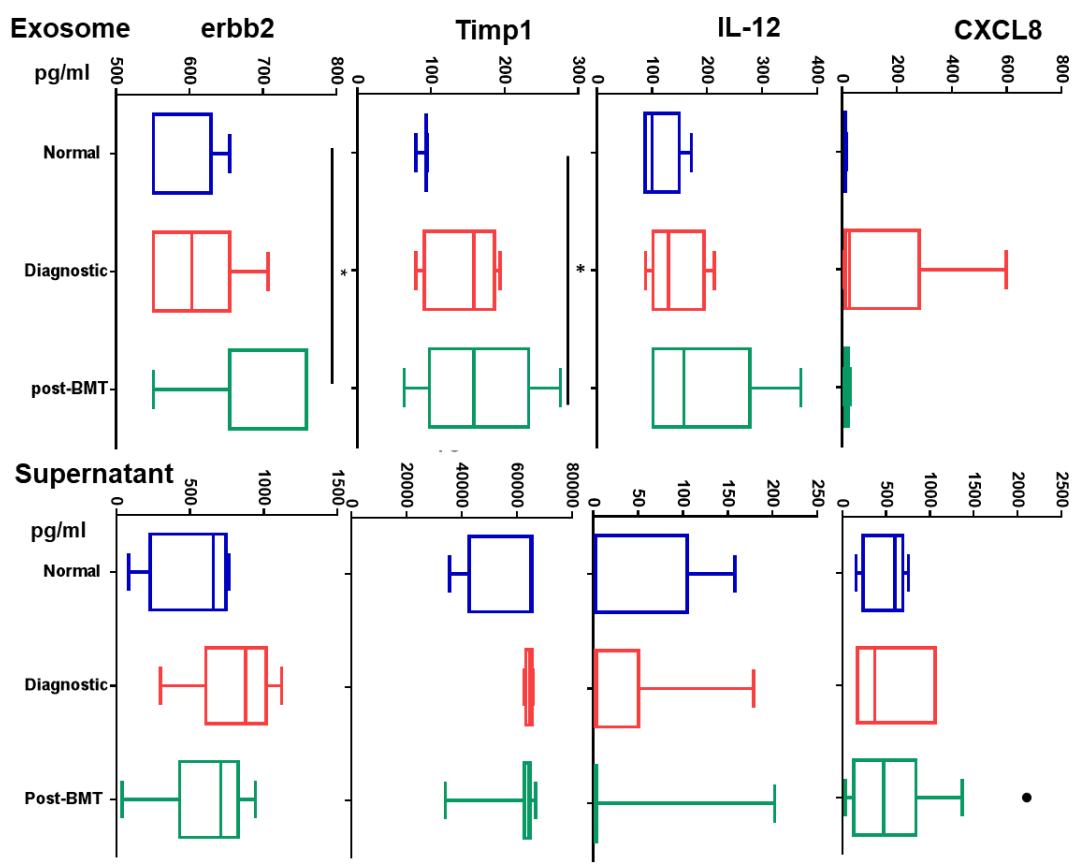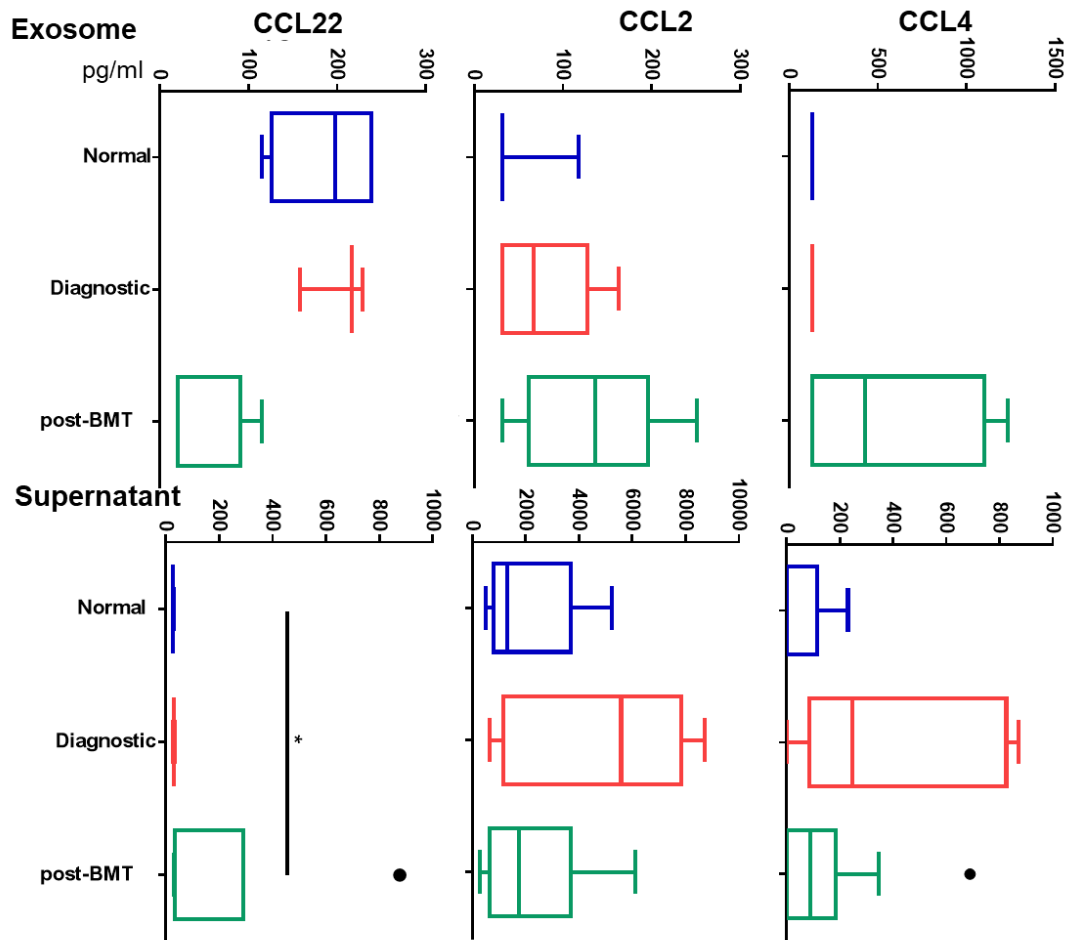
Figure 4. 10 Chart showing levels of chemoattract targets in normal, AML diagnostic and AML post-BMT MSC derived exosome (n=20) and supernatant (n=37) fractions as measured by Luminex immunoassay. *p = <0.05

4.3.2 Luminex Validation Assays

To confirm target expression identified by Luminex analysis I carried out western blotting and ELISA assays using target antibodies in order to validate some of the luminex results.

Exosome extractions derived from MSC cultures of normal (NBM), diagnostic AML (Diag) and post-BMT AML (BMT) origin were run on a western blot as outlined in Chaper.2 and probed for CD8 to confirm exosome status and two targets Her/erbb2 and ICAM-1 (Santa Cruz Biotech.,U.S.A) (Figure 4.11). Both targets showed an upregulation in post-BMT samples compared to an absence in normal, this was also observed within the diagnostic fraction for ICAM-1, these results reflect those seen within the Luminex analysis.
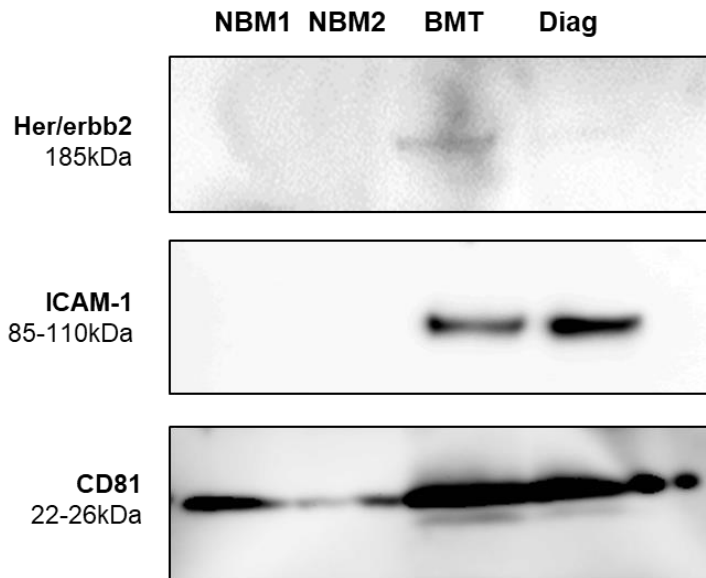
Figure 4. 11 Western blot showing MSC derived exosome preparations from primary normal bone barrow (NBM), AML diagnostic (Diag) and post-BMT (BMT) probed for exosome tetraspanin control (CD81), ICAM-1 and Her/erbb2.

MSC derived exosome extractions from leukaemic cell lines KG1a and Molm14 and all patient categories were used to carry out a permeabilized ELISA assay as outlined in Chapter.2 using antibodies ALIX, TIMP-1 and IGFBP3 as detailed in Table 2.1 at a concentration of 1µg/ml. The presence of ALIX was probed first as a control target for exosomes (Figure 4.12A) and showed similar levels in both primary and leukaemic cell lines as expected. An increased level of TIMP-1 was observed within the post-BMT samples compared to diagnostic and normal bone marrow which was significant (p=0.0109), and reflected those seen in the Luminex analysis (Figure 4.12B). A trend for higher levels of IGFBP3 could also be identified in the post-BMT also reflected in the Luminex results although this was not significant (Figure 4.12C).
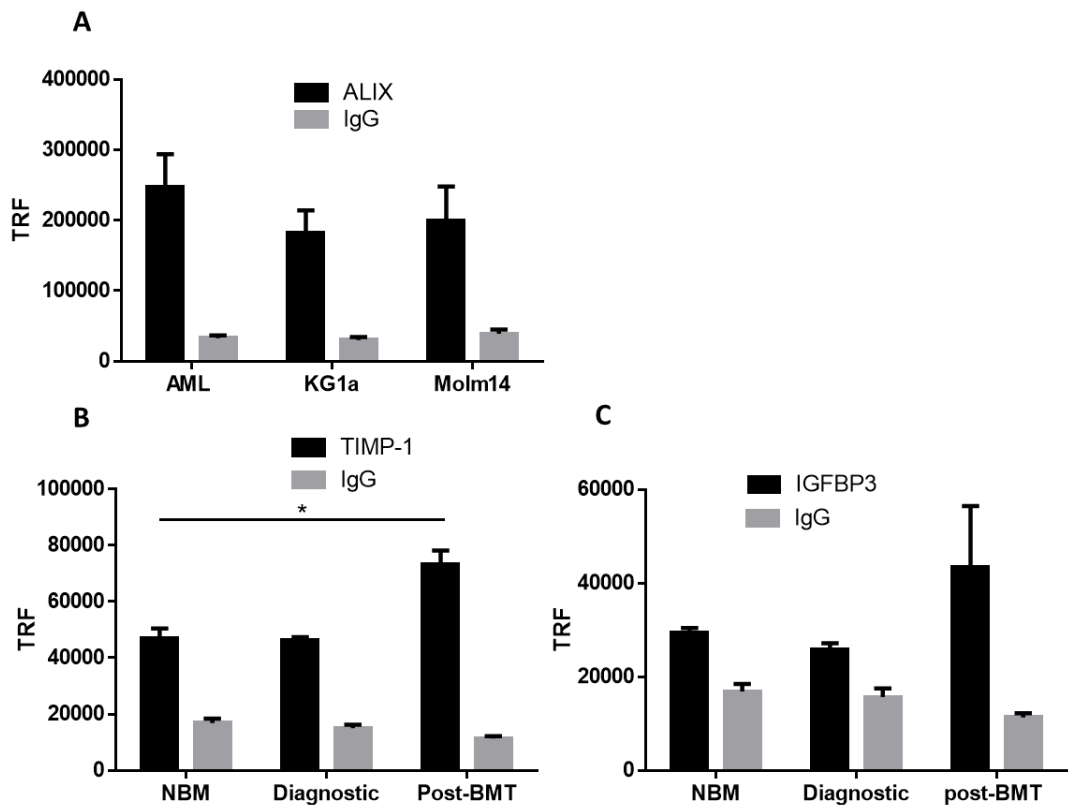
Figure 4. 12 Charts showing the results of permeabilized ELISA assays using MSC derived exosome preparations from primary AML and leukaemia cell lines (KG1a and Molm14) using exosome control target ALIX (A) and normal bone marrow (NBM), AML diagnostic and post-BMT for targets TIMP-1 (B) and IGFBP3 (C) (n=3) (*p = 0.0109).

4.3.3   TaqMan Assay

To assess the expression of two target mIRs commonly dysregulated in leukaemia, I carried out Real-Time PCR using a method as outlined in 4.2.2. Primer sets for mIR155 and mIR1246 were used and miRNA harvested from MSC derived exosomes samples from NBM, diagnostic and post-BMT analysed in order to compare target expression. For target mIR 1246 (Figure 4.13A) results showed a significant increase in expression of diagnostic (p = 0.0238) and post-BMT (p = 0.0357) compared to NBM. Expression of mIR155 (Figure 4.13B) also showed a trend of higher expression in diagnostic and post-BMT compared to NBM but this was not significant. Both targets showed high levels of variability within the post-BMT samples.
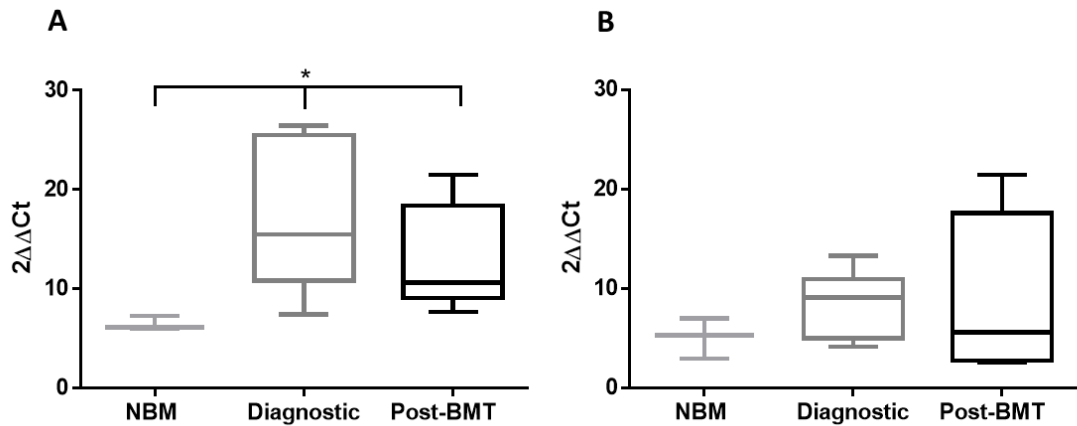
Figure 4. 13 Charts show the TaqMan RT PCR analysis of mIR1246 (A) and mIR155 (B) expression within MSC derived exosomal miRNA of Normal (NBM), AML diagnostic and post-BMT samples (n = 15), (*p = <0.05).

4.3.4 Functional Assays

To assess the functional behaviour of exosomes within co-culture, assays using the FLT3-ITD mutant leukaemic cell line MV-411 and exosomes derived from HS5 stromal cultures were set up adding 0-20 µg/ml of exosome preparations as outlined previously for 48h. Flow cytometry was used to assess cell viability and a dose dependent significant increase was observed upon addition of exosomes when co-cultured with HS5 stromal cells (p=0.0032) and HS5 cells alone (p=0.0007), no increase in cell number was seen when MV4-11 cells were cultured alone, (Figure 4.14A-C).

To check exosomal effect in another cell line I used the leukaemic p53 mutant cell line KG-1a increasing concentrations of exosome preps derived from HS5 stroma, diagnostic AML-MSC and NBM-MSC (0-60µg) Figure 4.14D-E. When KG-1a cells were cultured without stromal support (D) a small increase in cell proliferation was observed for all exosome additions although not significant, this was reflected in the presence of stroma (E). A dose dependent increase in cell proliferation was seen on HS5 cells alone with all exosome preparations used (F) (*p = 0.0120).
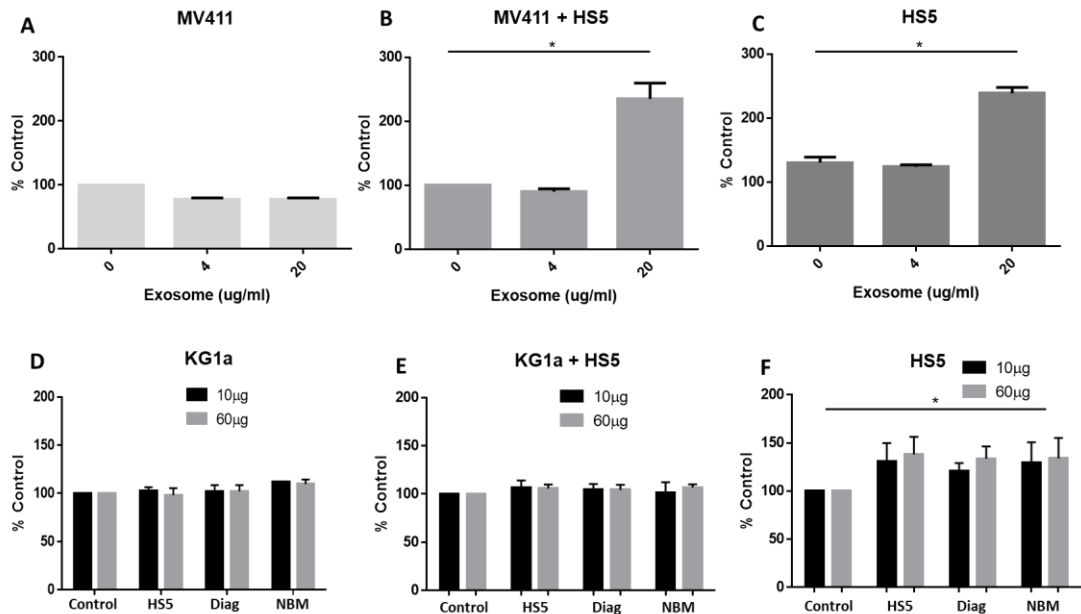
Figure 4. 14 MV411 cells were cultured +/- HS5 stromal cells over 48h with addition of 0 - 20μg of HS5 derived exosomes and proliferative effect assessed using flow cytometry of MV411 alone (A) *p=0.0002, co-culture with HS5 cells (B) *p=0.0032, HS5 cells alone (C) * p=0.0007 (n=3). KG1a cells were cultured over 48h +/- HS5 stromal cells with addition of 0 - 60μg of HS5, AML diagnostic and NBM derived exosomes and proliferative effect show on KG1a alone (D), co-culture with HS5 cells (E) and HS5 cells alone *p = 0.0120 (F) (n=3).

Although the effects of exosomes on KG1a cells were small, I investigated the potential effects on different sub-populations of cells in order to determine if the phenotype was more pronounced. Co-culture assays were harvested into adherent and suspension KG1a fractions and analysed by flow cytometry using CD45 antibody and viability staining as detailed in Chapter.2 (Figure 4.15). Results showed that upon addition of HS5 derived exosomes a small increase in cell proliferation was observed for both adherent and suspension fractions. When diagnostic and NBM derived exosomes were added there was no increase in cell proliferation within the suspension fraction, but a prominent increase within the adherent fraction was observed which was significantly higher within the diagnostic fraction (p = 0.0313) and an overall significant increase was observed following addition of exosome irrespective of origin (p = 0.0006). NBM derived exosomes showed an increased boost to cell proliferation in adherent cells compared to HS5, and diagnostic derived exosomes and concurrently suspension cell number was reduced, differences between suspension and adherent fractions were significantly different within the NBM exosome sub-set (p = 0.0286).
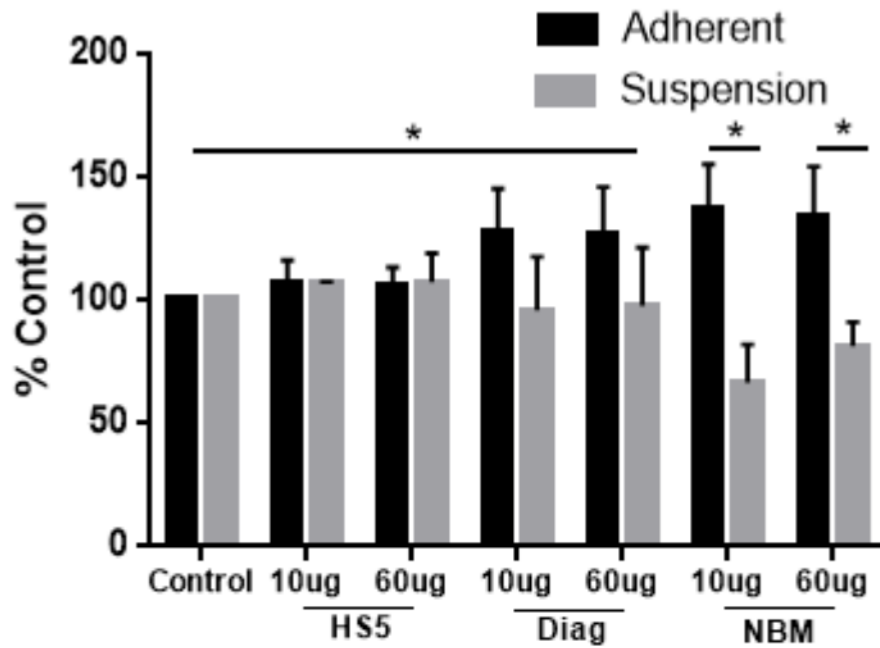
Figure 4. 15 KG1a cells were co-cultured +/- HS5 cells with HS5, AML diagnostic and NBM derived exosome at 0-60μg. Adherent and suspension fractions were separated post 48h incubation and proliferative effect assessed using flow cytometry (n=3) *p = 0.03.

Given the effects observed on KG1a adhesion I investigated the effects of exosomes on the migratory behaviour of KG-1a cells using a chemotaxis tissue culture plate as outlined previously. Total cell counts collected at the end of the assay showed no overall additional enhancement on KG-1a cells with addition of exosomes when cultured alone, but when in presence of stroma growth is further enhanced (Figure 4.16A). When cultured alone KG-1a cells showed limited migration and presence of exosomes did not have any additional effect (Figure 4.16B). When KG-1a cells were cultured in the presence of stroma they migrated towards the HS5 cells but again the addition of exosomes had no significant impact (Figure 4.16B).
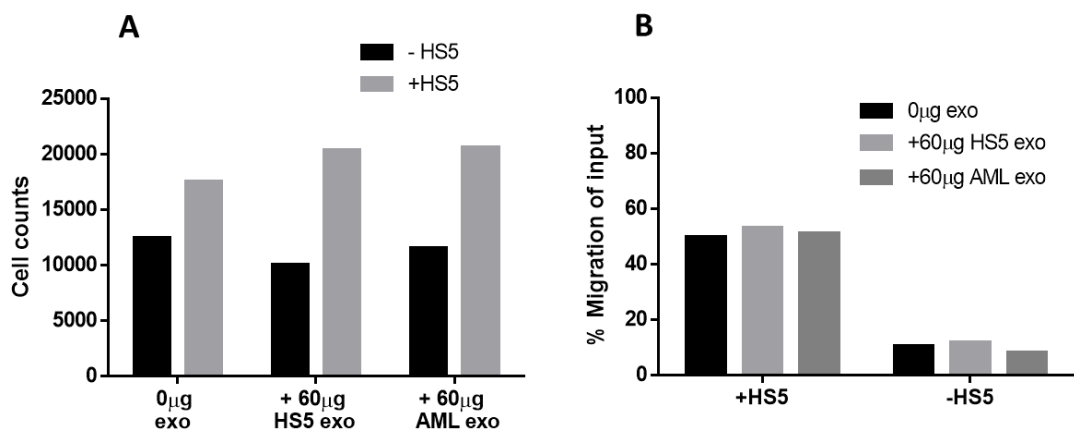
Figure 4. 16 Chart showing the migration of KG1a cells within chemotaxis tissue culture plate +/- HS5 stromal co-culture and the addition of 60µg of HS5 or AML derived exosome. The effect the addition of exosome had on cell proliferation (A) and the migratory capacity of the KG1a cells are shown (B).

In order to investigate the protective effects of exosomes, a tissue culture assay was set up using primary AML cells incubated with and without the addition of exosomes as outlined in 4.2.3.3. Following treatment with the drug Pacritinib; a FLT3 inhibitor, the EC_{50} of the cells incubated with exosomes was significantly increased showing the protective effect exosomes exert upon primary AML cells (Figure 4.17A).

ERK1/2 is a known key regulator of stromal:AML protection identified by previous work in our group and several known studies (Marrin et al. 2014; Yang et al. 2014). When primary AML cells were incubated +/- HS5 stromal co-culture and treated with increasing doses of Pacritinib, western blotting with ERK1/2 antibodies showed a dose dependent knockdown of ERK and pERK. This knockdown is less effective when AML cells are co-cultured with HS5 cells, showing the protective effects of stroma on ERK targeted drug treatment (Figure 4.17B).

KG-1a co-cultures +/-HS5 stromal cells were set up as previously described with the addition of a potent MEK inhibitor (MEKi) - PD0325901 (Sigma Aldrich, Dorset, UK) (Figure 4.17C). Following harvest of both supernatants and whole cell fractions, western blots were carried out for ERK1/2 detection. As expected, KG-1a whole cell lysate (WCL) showed the presence of ERK and with addition of MEKi this was knocked down. ERK could not be detected in KG1a derived exosomes. When KG-1a was in stromal co-culture with HS5, ERK was present within the control fraction of both the whole cell lysate and the exosome fractions, suggesting co-culture stimulates ERK containing exosomes. This increase in exosomal ERK was knocked down with MEKi treatment in both the cell and exosome fractions. CD81 was used as a marker for exosome and GAPDH as a housekeeping marker for exosome and WCL.
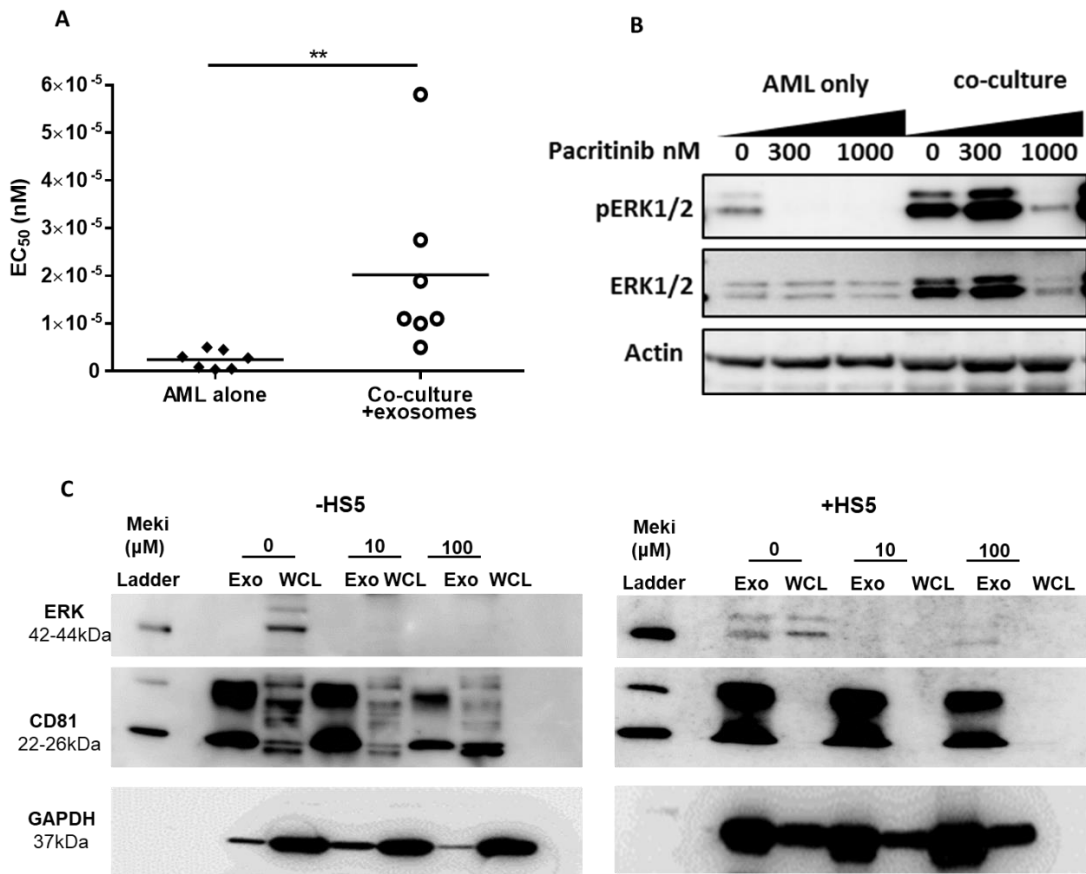
Figure 4. 17 Chart showing the effect of FLT-3 inhibitor (Pacritinib) on primary AML cells co-cultured +/- exosome **p = 0.0026 (A) Western blot showing the dose dependent effect of Pacritinib on MV411 cells cultured +/- HS5 cells on ERK expression (B). Western blot of KG-1a cells incubated +/-HS5 cells and MEK inhibitor (MEKi) PD0325901 added at 10μM and 100μM. Exosome (Exo) and whole cell fraction (WCL) were extracted post 48h incubation and probed using ERK (42-44kDa) CD81 (22-26kDa) and GAPDH (37kDa) antibodies (C).

In order to confirm my observations in primary patient material tissue culture assays were set up using HS5, NBM, diagnostic and post-BMT derived exosome preparations to assess functional effects (Figure 4.18A-D). Results across a cohort of four patient samples showed a significant increase in cell proliferation following addition of exosomes. The source of exosome preparation was extraneous to proliferative effect.
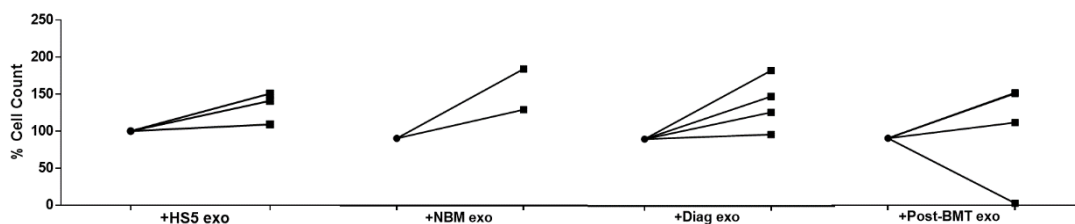
Figure 4. 18 Primary AML cells (n = 4) were co-cultured with HS5 stromal cells in the presence of 60µg/ml HS5, normal bone marrow (NBM), AML diagnostic and post-BMT derived exosomes. Cell proliferative effects were analysed using flow cytometry following 48h incubation.

To investigate the effects of exosome addition compared to stroma alone in primary AML samples, Figure 4.19 shows changes in proliferative response in three primary AML samples. A significant increase in proliferation was observed for all co-cultured with HS5 compared to control alone. Exosome addition gave variable responses amongst primary AML blasts. AML 1 and 2 both showed that although exosomes increase proliferative effect, the impact of the stromal effect itself is stronger than exosomes at encouraging growth. AML 3 however shows that even in the presence of a modest stromal effect, the addition of exosomes can further boost the proliferative response.
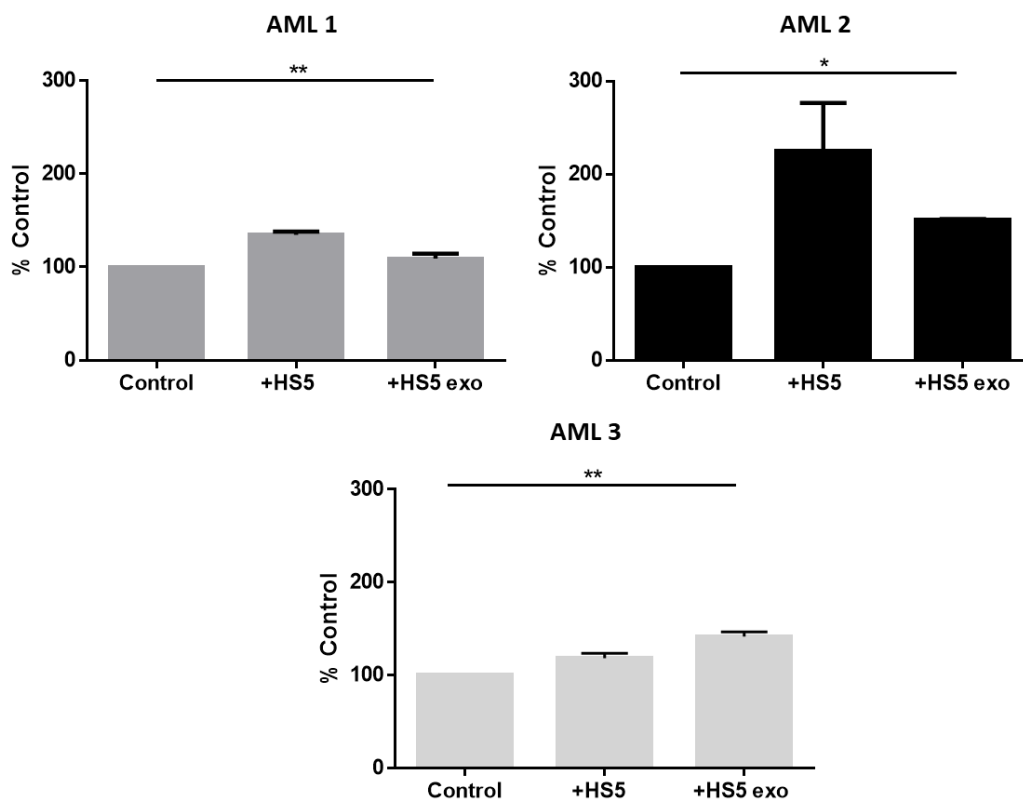
Figure 4. 19 Primary AML samples were co-cultured +/- HS5 cells and +/- 60µg HS5 derived exosome over 48h to compare effects of stromal vs exosome proliferative effect (AML1 and AML 3 ** p=0.0036, AML 2 * p=0.0464) (n=3).

The effects of increasing stromal exosome concentration on primary AML blasts was carried out in 48h co-culture assays +/- HS5 and the addition of 0-120µg of post-BMT derived exosomes. Flow cytometry was used to separate the adherent and

suspension fractions and measure cell proliferation (Figure 4.20A) The effect of adding BMT derived stromal exosomes into culture produced a significant increased proliferative effect, even without stromal support and within the suspension fraction of co-cultured plates in a dose dependent manner. Adherent fractions showed minimal increased effects at high doses of exosomes suggesting adhesion is not increased with BMT exosome preparations.

Furthermore, the effects of NBM, diagnostic and post-BMT derived exosomes on two AML samples alone and in co-culture showed a trend for increased cell proliferation following all exosome additions, although AML 2 showed a more pronounced effect when exosome and stroma were present together (Figure 4.21).
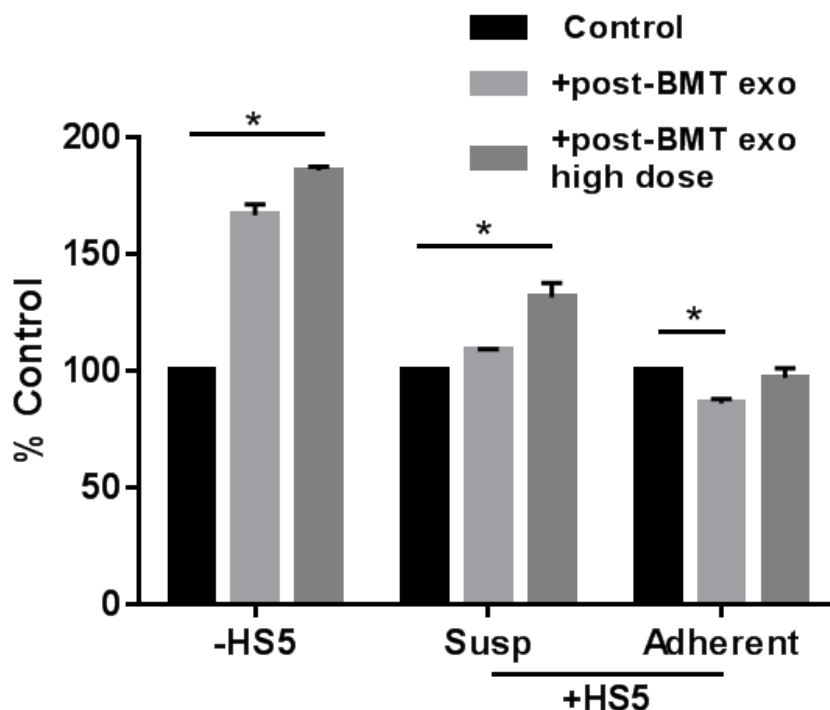
Figure 4. 20 Primary AML sample was incubated +/- HS5 stromal with 60µg of AML post-BMT (+Post BMT exo) and 120µg AML post-BMT (+post BMT high dose) derived exosome incubated in culture for 48h. Suspension and adherent co-culture fractions were separated and analysed by flow cytometry. Significant difference was observed for both co-culture and alone between control and addition of exosome (*p=<0.0001, n=3)

AML 1

AML 2
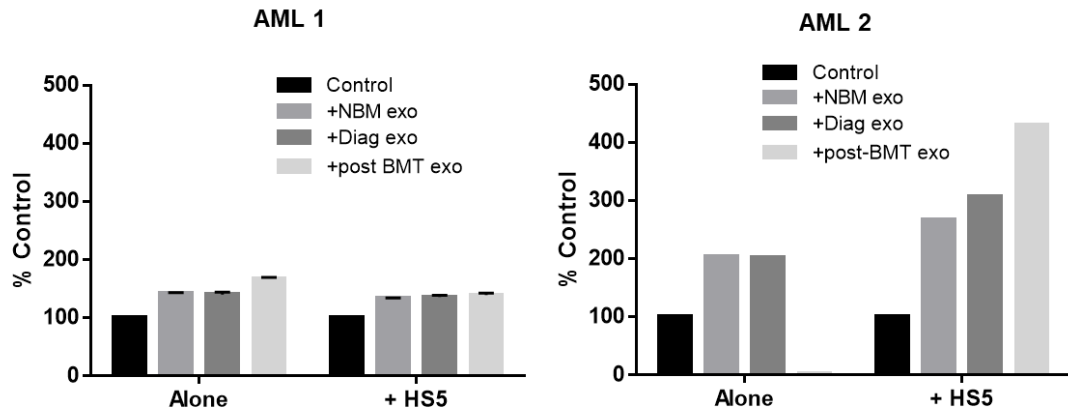
Figure 4. 21 Primary AML samples were co-cultured +/- HS5 cells with 0-60µg of NBM, AML diagnostic and AML post-BMT derived exosomes added to culture. Post 48h incubation cell proliferation was assessed using flow cytometry (n=3).

The effect of exosome addition on primary AML suspension and adherent fractions was investigated (Figure 4.22) and within the suspension fractions (A-C) addition of exosome was seen to enhance cell proliferation in most cases, an exception is seen for one AML (Figure 4.22A). Adherent fractions showed no proliferative benefits from addition of exosomes (Figure 4.22D-F).
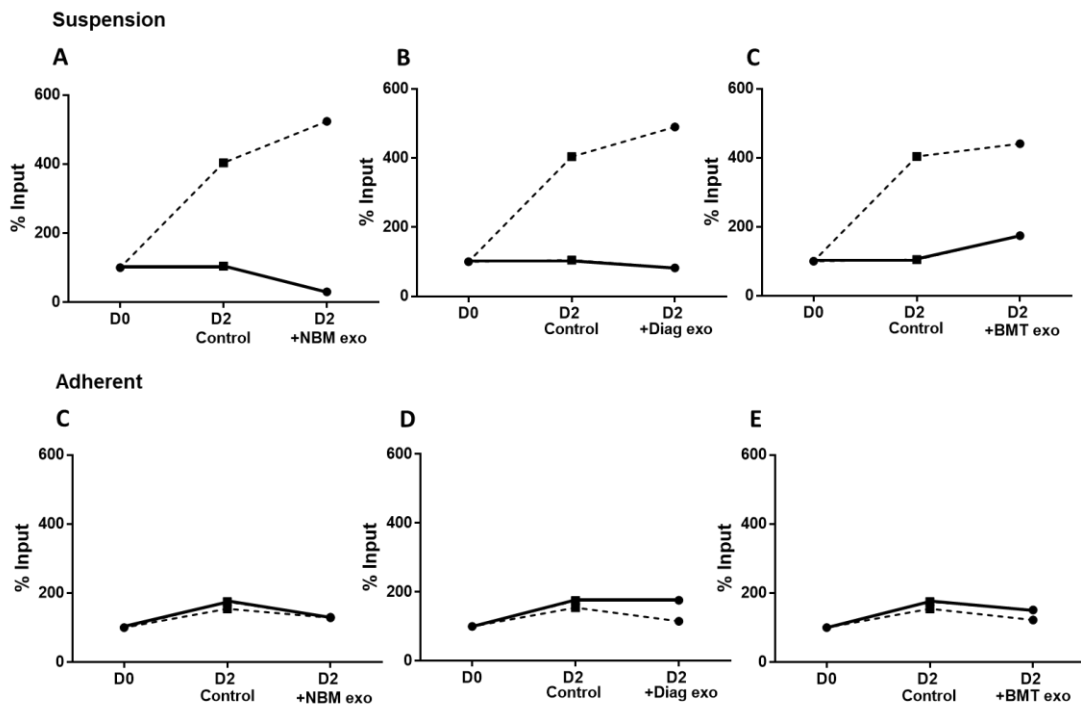
Figure 4. 22 Primary AML samples were co-culture with HS5 and incubated for 48h with 60µg of NBM, AML diagnostic and AML post BMT derived exosomes and separated into suspension (A-C) and adherent (D-F) fractions. Analysis by flow cytometry plotted at day 0 (D0) and day 2 (D2) post exosome addition (n=3).

Finally, the effects of exosome addition on HS5 cells were analysed within primary AML co-culture and found that it provided minimal proliferative support, post-BMT derived exosomes were the only exosome sub-type to increase cell proliferation, although not significantly (Figure.4.23).
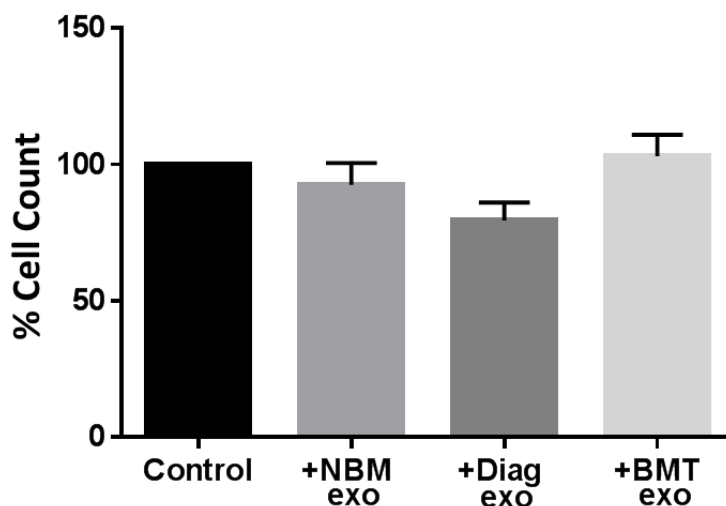
Figure 4. 23 Chart showing the effect of adding 60µg of NBM, AML diagnostic, AML post-BMT derived exosome on HS5 stromal cells over 48h incubation period (n=3).

In summary:

- Luminex profiling identified changes in target expression between NBM, diagnostic and early-mid and late post-BMT, highest levels observed in diagnostic samples
- Overall lower expression of targets in exosome compared to supernatant
- Post-BMT samples showed high expression of immune related targets
- Expression of leukaemic related mIRs showed an increase in levels within diagnostic and post-BMT samples compared to NBM.
- MV411 cell lines show increased proliferative effect upon addition of exosomes in culture.
- Exosome addition to KG1a cell line co-culture showed a small increase in proliferation and migration which increased on stroma within the adherent fraction.
- Addition of exosomes within KG1a stromal co-culture induced a survival advantage involving ERK signalling

- Primary AML cells show an increase in proliferation upon addition of exosomes which varies between samples
- Exosome addition shows more effect on proliferation within suspension fraction of stromal co-cultured primary AML cells.

4.4   Discussion

Luminex analysis showed that cytokine profiles of exosome fractions contained fewer targets at detectable levels compared to the much more diverse cellular derived content of the supernatant fraction. Published work comparing the cytokine content of exosomes to that of their cellular counterpart is sparse, as far as I am aware this is the first time this has been shown in AML. There are studies that show differences between plasma content and equivalent exosome cytokine levels, and this is reported to be target dependent with substantial differences seen between both (Prieto et al. 2017; Kodidela et al. 2018).

A study involving head and neck cancer compared protein content between exosomes and equivalent cell lysate from various cell lines, and found that the exosome profile did not always correspond to that of the parental line but, overall expression levels were lower within the exosome samples, agreeing with my observations (Ludwig et al. 2018). A likely explanation for lower levels of protein expression within the exosome fractions could be due to their small size and very heterogeneous content compared to the secretory samples. The molecular content of exosomes originating from different types of cell and biological fluids has been identified to contain proteins, nucleic acids, lipids, metabolites as well as thousands of different macromolecules (www.exocarta.org), this is all contained within the exosome preparation and could impact on downstream analysis and result in a lower level of expression compared to the less heterogeneous secretory sample.

When cytokine levels were compared between exosomes originating from AML cells to normal bone marrow, a heightened expression of targets was seen in the diseased cells. This could perhaps be explained by the important role exosomes originating from MSC cells play in remodelling the bone marrow microenvironment into a malignant niche. It is known that MSC gene expression and functionality in AML is different to that from healthy individuals, and exosomes have been shown to play a major role inducing these microenvironmental changes in AML (Barrera-Ramirez et al. 2017). Consequently, it is probable that exosomes originating from AML derived MSCs would show higher expression of numerous targets involved in bone marrow

microenvironment remodelling, compared to normal derived MSCs which are only likely to play a role in maintaining homeostasis within a stable BM-niche.

Cancer derived exosomes are known to be enriched in molecules which help support disease proliferation, metastasis and immune surveillance avoidance (Hong et al. 2014a), exosomes derived from MSCs within a malignant microenvironment would show similar enrichment which could further explain the high levels of target protein detected.

The difference in overall abundance levels of targets and their function between supernatant and exosome is perhaps due to the heterogeneity of supernatant content compared to the more specific and targeted content of exosomes. It is known that exosomes function as communication vehicles and their contents can differ from that of the parental cell, making it logical that the signalling proteins it transports would be more specific to a function of the environment it resides (Willms et al. 2016).

Further investigation into the breakdown of Luminex analysis using hierarchical charts shows a clear division of expression of targets within exosomes, there was much higher expression in NBM and diagnostic samples compared to both early-late post-BMT. One of these targets was C-kit which when analysed independently showed significantly lower expression in the majority of post-BMT samples. C-kit mutations in t(8;21) AML are monitored at MRD level for relapse in post-transplant cases as mutated cases are more likely to relapse compared to unmutated. The lower levels of C-kit expression seen here in post-BMT could signify a positive clinical outcome (Ossenkoppele et al. 2016), and may reflect a novel level of regulation from the cell surface into exosomes.

Overall expression levels of targets within the exosome fraction (Figure 4.6) identified VCAM-1 as the most highly abundant target present within all categories of sample type and highest within the NBM. VCAM-1 is a protein which acts as a mediator of the adhesion between lymphocytes, monocytes, eosinophils and basophils to the vascular endothelium where it is also involved in leukocyte/endothelial cell signal transduction. It is unsurprising that it's seen in such high levels in NBM, as it is an important requirement within a normal functioning bone marrow niche.

The down regulation of VCAM-1 observed in the post-BMT has previously been observed within work involving ALL, bone marrow stromal cell cultures post - transplant showed significantly reduced expression compared to normal donor bone marrow. Further investigations show a correlation between B-cell precursor growth and adhesion and CD106 expression, it is thought that this could be why recovery is

delayed post-transplant, as the capacity of transplanted stromal cells is impeded by low B-cell lymphopoiesis (Dittel and LeBien 1995).

Another adhesion molecule ICAM-1 is highly expressed in all sample types although lower levels were observed in NBM and later stage post-BMT. ICAM-1 binds to the surface of leukocytes and becomes induced under inflammatory conditions aiding the migration of leukocytes and activation of T-cells (Dustin et al. 1986), the higher levels observed in diseased and early post-BMT could reflect the heightened immunological environment expected in these samples compared to normal. Late post-BMT reflects a stabilization of the inflammatory environment towards normal bone marrow levels along with an increase in adhesion related molecules suggesting a recovery post-transplant, the stromal microenvironment becoming more hospitable to transplanted stromal cells forming a niche with the aid of various adhesion molecules.

Early post-BMT exosomes showed high levels of IL4 and IL10, these are both involved in immunological function and within an early post-BMT environment you would expect immune response to be heightened during immune constitution, which varies in timescale from patient to patient. Recent research has identified IL-4 as a selective inhibitor of AML cell growth and survival, which could be a useful aid within a post-BMT environment to suppress minimal residual disease and could be used for monitoring purposes. (Pena-Martinez et al. 2018).

IGFBP-3 was more highly expressed within post-BMT exosome samples compared to other sample stages. IGFBP-3 functions as an aid to cell survival and differentiation and is dysregulated in many cancers. IGFBPs have been identified as playing an important role in tumour proliferation, and studies involving adolescent AML and ALL have shown that important changes take place within the IGF (insulin-like growth factors) system following haematopoietic stem cell transplant, as they play an important role in the proliferation rate of transplanted bone marrow. This could help explain the heightened levels observed in post-BMT (Dawczynski et al. 2003).

Further analysis of targets identified within the exosome fraction (Figure 4.7-10) showed that within post-BMT samples high levels of targets mostly associated with immunological and chemoattract functions were observed, and NBM showed a higher expression of targets associated with survival/differentiation and adhesion. This observation was further confirmed within pie chart analysis of target functional groups, (Figure 4.6) early post-BMT showed very clearly that the highest expression of targets were those associated with an immunological function, and interestingly mid-late post BMT reverts to a profile more associated with survival and adherence seen in

diagnostic and NBM. This bias towards immunomodulating targets seen early post-transplant suggests that exosomes might be involved in the transfer of soluble factors, which have been reported to manipulate T-cell function that could lead to the aid of a hostile or supportive environment for AML (Lamble and Lind 2018).

Comparison between exosome and supernatant expression profiles (Figure 4.8-10) were very different, and only showed a similar profile within the targets associated with adhesion and targets BMP-4 and Protein-S both associated with survival/differentiation. Apart from these exceptions the expression profiles of exosomes were very different to supernatant between patient sub-categories.

This was further confirmed within the pie-chart analysis (Figure 4.6). Results showed that supernatant expression profiles of NBM and diagnostic samples showed a dominance of survival/differentiation which differed greatly to exosome profile of adherence. Post-BMT however, showed more similarities between exosome and supernatant. NBM and diagnostic profiles within the supernatant fraction showed very high levels of osteonectin, the highest of which were seen in diagnostic samples and absent from early post-BMT and at low levels within mid-late-BMT. Osteonectin is involved in cell differentiation and regulates the adhesion of osteoblasts and stromal support, and has been strongly implicated as a poor prognostic marker of AML. Research has shown that it promotes leukaemic cell growth and could explain it's prominence within the diagnostic samples (Alachkar et al. 2014).

The miRNA content of MSC derived exosomes for all sample sub-sets was assessed for the expression of mIR155 and mIR1246 (Figure 4.13), these targets were selected as both have been shown to be exported in exosomes and correlated with presence of leukaemia (Hornick et al. 2015).

My work showed that expression of mIR1246 was significantly increased in samples of post-BMT and diagnostic origin compared to NBM. This agrees with previous work that has shown an increase in association with presence of leukaemia (Hornick et al. 2015). Previous work using an AML-engrafted mouse model showed that mIR1246 expression from exosomal miRNA increased in correlation with disease progression (Abdelhamed et al. 2019), this could help explain the large spread of data I observed within the post-BMT samples, perhaps reflecting the heterogeneity of disease recovery post-transplant. Previous work showed that EV's enriched with mIR1246 were transferred from AML cells to HSCs leading to DNA damage and dysregulation of the protein kinase S6RP and an associated induction of p53- dependant quiescence (Abdelhamed et al. 2019). The quiescence imposed upon residual HSCs

within the leukaemic niche could be a good indicator of MRD and another explanation for the varying levels of expression I observed post-BMT. The link between mIR1246 and dysregulation of p53 has been seen in numerous other studies including the B-cell dysfunction within systemic lupus erythematosus and the inhibition of the Down Syndrome associated gene DYRK1A expression which has been seen to occur via p53 induction of mIR1246 (Zhang et al. 2011; Luo et al. 2015).

Another study showed an association between increased oesophageal squamous cell carcinoma with mIR1246 expression and interestingly when they compared cellular and exosomal expression levels they found much higher levels within the content of exosomes, indicating the important secretory role exosomes play (Takeshita et al. 2013). This study highlights the importance of assessing exosome mIR content as opposed to cellular as it could provide more accurate information regarding disease progression.

mIR155 has been associated with dysregulation with many cancers (Tili et al. 2010; Donnem et al. 2011; Lanczky et al. 2016), in AML it is thought to be involved in regulating genes which play active roles in promoting leukaemogenesis (Hornick et al. 2015). My results agree with previous research and show a trend of increased expression within the diagnostic and post-BMT samples compared to NBM.

Both mIRs profiled here have shown in previous studies an association with B-cell dysregulation, mIR155 transfer from leukaemic cells via gap junctions has been shown to directly transform healthy B-cells to a malignant form (Tili et al. 2009). It has been reported that mIR1246 has been linked to p53 expression resulting in abnormal B-cell function (Luo et al. 2015). The increased expression observed of both mIRs in post-transplant samples along with increased levels of immunomodulatory targets highlighted within the Luminex analysis provides more evidence of the role exosomes play in mediating immuno-modulatory effects within a post-transplant environment.

The functional effects of exosomes within a cell culture environment varied depending on cell source (Figure 4.14). When using MV411 cells a significant increase in proliferative effect was observed when exosomes were added to a stromal co-culture environment. This effect was reduced for KG-1a cells, with only a small proliferative advantage observed which was not dependent on exosome source, illustrating that exosomes can offer a proliferative advantage across different cell types irrespective of parental cell, this agrees with previous research which has shown that cancer cells can use exosomes to manipulate their microenvironment to gain survival advantages (Clayton 2012; Corrado et al. 2014; Zhou et al. 2016).

Further investigation into exosomal effects within KG-1a stromal co-culture (Figure 4.15) revealed addition of exosomes had an increased proliferative effect on cells within the adherent fraction compared to those in suspension, suggesting that the increase in cell number was driven by adhesion rather than a proliferative effect of exosomes (Taverna et al. 2014; Tang et al. 2016).

The effect of exosomes on the migration of KG-1a cells (Figure 4.16) showed minimal advantage, and the presence of stroma created the biggest drive in migration, this could be explained by the higher concentration of various soluble factors and cytokines secreted by the HS5 stromal cells in culture compared to the relatively small exerted by exosomes. The small increase in total cell numbers upon addition of exosomes within the stromal environment show that although exosomes do have an impact on cell proliferation, it is minimal compared to the effect of stroma in this context and not enough to stimulate a migratory response. This is likely to be cell type dependant as a previous study involving CML showed that cell migration was stimulated by exosomes but it was the exosome cross-talk with stromal cells that stimulated the release of IL-8 and increased migratory effect, this could help explain why migration is only seen here when in presence of stroma (Corrado et al. 2014).

The exosome induced cell survival advantage in KG-1a cells was further investigated using western blotting to analyse pro-survival ERK signalling in exosomes of KG-1a cells in co-culture (Figure 4.17). Stromal induction of ERK in exosome fractions was detected in co-culture and could be abrogated through treatment with MEK inhibitors. ERK1/2 signalling is known to become frequently activated in AML co-cultures and provides pro-survival functions associated with drug resistance (Lunghi et al. 2003). The source of the ERK expressing exosomes is unknown, it is possible they originate from HS5, KG1a or both cells but in order to determine this, it would require separation of these post-culture which is currently limited by technical difficulties. Irrespective of exosomal origin, those released within a stromal co-culture environment are different to those within a single culture system and induced a survival advantage in KG-1a cells.

The functional effect of exosomes on primary AML cells was different to that seen in cell lines (Figure 4.18). A significant increase in proliferation was observed upon addition of exosomes which was AML specific and although stromal support did offer a proliferative advantage to AML cells this was not always stronger than the effect of exosome alone, this variability could be as a result of the heterogeneous nature of AMLs which have been shown to exhibit inherent cytokine independence deeming

them less reliant on stromal support and survival in co-culture (Yang et al. 2014; Huang et al. 2015).

The effect of exosomes within primary co-culture showed a more proliferative effect exerted on cells in suspension compared to adherent, this was shown very clearly within the suspension fraction of one AML sample in a dose dependent manner (Figure 4.20). This could be due to the larger cell surface area and increased possibility of interaction of cells in suspension with circulating exosomes. Those within the adherent fraction would become immobilized when connected to the stromal layer and direct cell to cell contact with stromal cells could provide the adherent cells with sufficient support. A previous study involving breast tumour cells showed that increased exosome uptake and arrangement on cell surface enhances call adhesion and proliferation (Koumangoye et al. 2011). The presence of exosomes within a co-culture environment could be stimulating more adhesion and proliferation, which is why we see an enhancement for both cell lines and primary cell culture. Cell lines may show a reduced uptake of exosomes compared to primary cells due to the downregulation of numerous cell surface receptors that takes place when cells are immortalised, cell lines are characteristically more self-sufficient and so don't require the same level of support supplied to primary cells via exosomes. A way to test this theory would be to culture cell lines within serum starved conditions and measure the effect of exosome addition.

Future plans also include running matched plasma samples from AML patients. Plasma will contain a plethora of molecules originating from numerous cell subtypes so would be interesting to see whether the dominance of a cell differentiation related function is reflected, and a commonality shared between the malignant or healthy supernatant and exosome fractions. Cohorts could also be examined for molecular/morphological relapse in order to determine whether candidates previously identified segregate with these groups at earlier time points.

5 Chapter 5 Discussion

The study of extracellular vesicles is a popular field of research with much still to discover, we know that they play an extremely important role in many aspects of human disease and have potential use as a monitoring tool, biomarker and therapeutic. The aim of this study was to investigate how important extracellular vesicles are as promotors of residual AML survival and better understand their role in creating a hostile microenvironment that favours disease survival. Identification and characterisation of differences between exosomes derived from diseased, normal and post-BMT AML has the potential to identify biomarkers and early relapse.

Characterisation of exosome material highlighted significant differences between patient sub-sets of MSC derived exosomes. Diagnostic AML samples showed an elevated level of exosome production compared to other sample types, which corresponds to disease presence and is significantly higher than normal bone marrow production which is to be expected as similarly seen in previous work (Taylor and Gercel-Taylor 2008; Rosell et al. 2009; Jenjaroenpun et al. 2013). Interestingly early and late post-BMT samples displayed different levels of exosomes, early-BMT more similar to the lower levels seen in NBM and late-BMT showed a large spread of data unlike any other sample type. These observations suggest that for some patient's recovery post-transplant is not stable, an important finding which warrants further investigation with an expansion of sample numbers and correlations with clinical presentation of relapse or graft failure.

Recent work has confirmed that exosome secretion from AML blasts leads to a complete remodelling of the bone marrow niche into a leukaemia permissive environment while suppressing the growth of normal haematopoiesis, this reflects the characterisation work I carried out on MSC derived exosomes which showed higher numbers within diseased and post-transplant samples compared to normal (Kumar et al. 2018). The suppression of normal haematopoiesis via blast derived exosomes has also been seen in work carried out by Namburi et al which showed the presence of exosomes in AML patients in CR suppressed normal haematopoiesis (Namburi 2018). This could help explain the variability seen in post-BMT as these samples could contain BM-ME at different stages of recovery and the higher levels of exosomes observed could signify the sustained repression of normal functional behaviour of the HSCs residing there. Work carried out investigating the impact of EVs within the tumour microenvironment of breast cancer showed that a transfer of

miRNA via EVs transformed surrounding normal fibroblasts into cancer supporting cells, this reflects another route by which exosomes could become involved in transforming a normal BM-ME into a malignant one (Vu et al. 2019).

The large variability I observed within the late-BMT sub-set of samples could also be an early indicator of patient transplant success or failure, but considering the heterogeneity of AML this could indicate the variation in time taken for each patient to recover normal homeostasis within the bone marrow microenvironment following transplant. It is known to take up to two months to recover haematological function (Pavletic et al. 1998), and up to 30 days for innate, and up to 1 year for adaptive immune reconstitution (Ogonek et al. 2016) but this is variable between patients.

Previous work involving a murine heart transplant model showed that monitoring of exosome numbers can give an indication of acute rejection, this study showed that an increase in donor exosomes which were identified by labelling with anti-H2-K^d-antibody and assessed using a nanoparticle detector showed a correlation with a reduction in graft rejection (Habertheuer et al. 2018). The positive link between exosome presence and increased transplant success has been reported in numerous in vitro studies which show that exosomes including those originating from Treg cells, suppress inflammatory response and allograft tolerance (Peche et al. 2006; Kim et al. 2007; Agarwal et al. 2014). These could help explain observations in my work and give an indication of graft progression within patients. The effect of exosomes on transplant is not always positive, a recent *in vivo* study showed that donor exosomes transferred MHC-complex to antigen presenting cells which initiated skin, heart and islet graft rejection (Marino et al. 2016). It is clear that exosomes are involved in many processes, some will have a positive impact and others a negative on transplant success.

Previous work has shown that the miRNA contained in exosomes can differ to that of the parental cell but in the case of cancerous cells can provide information regarding potential gene dysregulation, which correlates with disease progression making it an attractive potential diagnostic tool (Mittelbrunn et al. 2011; Hornick et al. 2015; Barrera-Ramirez et al. 2017). I found that miRNA content of exosomes was elevated in diagnostic samples compared to normal and post-BMT, it has previously been reported that the amount and composition of exosomal miRNA differs between diseased patients and healthy individuals (Zhang et al. 2015). A recent study used a human-into-rat xenogeneic heart transplant model to investigate the miRNA content of exosomes produced by transplanted cardio related cells, they isolated exosomes

from blood and compared it to exosomes extracted from the same cells cultured *in vitro,* and showed that their miRNA contents differed, the *in vivo* derived exosomes contained miRNA linked to miRNA recovery (Saha et al. 2019). Many cancer studies involving the profiling of exosomal miRNA content has shown those originating from malignant origin contained higher concentrations of differentially expressed miRNA compared to healthy individuals (Taylor and Gercel-Taylor 2008; Li et al. 2016; Bhome et al. 2017). A study involving breast cancer showed that exosomes derived from BM-MSC cells contained much higher levels of miRNA compared to normal cells and specifically overexpression of mIR23b that induced dormant phenotype leading to promotion of breast cancer cell dormancy within a metastatic bone marrow niche (Ono et al. 2014). Previous work involving AML cell lines showed an enrichment of exosome derived miRNA and *in vivo* mouse model work showed that miRNA from exosomes could help predict relapse in AML (Huan et al. 2013; Hornick et al. 2015). These studies illustrate how the microenvironment of malignant and transplanted cells has a direct impact on exosome number, concentration and content which is mirrored in the differences I have observed in AML and indicates the effect of genetic material transfer via exosomes can have and transform the microenvironment of these cells and improve their survival.

Several studies have reported the presence of miRNA in AML derived exosomes and the effect this has on compromising haematopoiesis and regulating communication within the tumour microenvironment (Hornick et al. 2016), the preliminary mIR profiling I carried out reflected this showing an increased expression of mIR1246 and mIR155, both associated with leukaemic state. Going forward I would like to profile these AML patients to try to identify corroborating changes in gene expression, similar work has been carried out by Barrera-Ramirez et al, but in a different context (Barrera-Ramirez et al. 2017).

My work showed no correlation between MSC size with exosome and miRNA yield indicating the differences observed were indeed disease stage specific and not simply a product of the area of the cells, to the best of my knowledge this is the first time a report on RNA yield of MSC derived exosomes has been made. Further studies to quantify the degree of basal differentiation (osteoblast, adipocyte, primitive) of the different stromal layers would be of interest to determine whether heterogeneity has impact on exosome production, this work will be carried out in subsequent projects. Increased exosome number did show some association with an increase in miRNA yield, but as previously stated this is not thought to be necessarily important, as it is known that only a fraction of miRNA present within the parental MSC goes on to

become transferred by the MSC exosomes and the content of miRNA is heavily influenced by the microenvironment of the cells, therefore the amount of miRNA being carried as cargo may not reflect the downstream mechanism of action generated (Bell and Taylor 2017).

A recent publication involving MSC derived exosomes commented on the lack of reported information to date on RNA yield from MSC exosomes (Toh et al. 2018). One of the challenges within the extracellular vesicle field is producing adequate exosome yield, and although sample size was small I showed significant differences in exosome numbers and yield based on disease stage, I believe my work has gone some way to investigate exosomes derived from MSCs. An expected standard concentration of exosome protein and miRNA harvested from MSCs still requires further standardization of the exosome isolation process and is an area of ongoing concern outlined by the International Society for Extracellular Vesicles.

I previously discussed in Chapter 3 that the post-BMT samples that showing higher levels of exosome release could be an early indicator of GvHD. Recent studies have shown the use of exosomal miRNA profiles in helping to make a molecular diagnosis post-transplant, they showed that certain mIRs associated with inflammation and immune response were upregulated within patients showing late onset GvHD and that exosomal miRNA could also have a potential use as a biomarker for GvHD prediction (Yoshizawa et al. 2018). Whilst this study examined a small number of relapse samples, it would be interesting for future work to follow up on serial patient samples that showed higher levels of exosome release post-BMT to investigate a link to relapse or GvHD. As GvHD is present in approximately 30% of samples at any one time point post-transplant, further investigation is needed with larger sample numbers enabling disease progression and associated exosomal changes to be tracked.

Recent research has brought into doubt the commonly held belief that miRNA is the driver of exosomal functional behaviours, they state that it is more probable that protein content of exosome cargo is more likely to propel mechanism of action, it is suggested that the quantities of miRNA present in MSC derived exosomes are not sufficient to bring about a biologically relevant response unlike proteins which they illustrate are more commonly present at high enough levels to illicit a mode of action (Toh et al. 2018). I investigated the protein content of exosomes using Luminex analysis and found there was an overall higher expression of target protein levels within AML patients compared to healthy individuals, the experiment was carried out using normalised exosome numbers for each patient analysed so this increase in

protein expression signifies a difference in protein loading of exosomes as opposed to increased exosome release which has been observed in many other studies. This increase in protein levels of cancer derived exosomes has been seen in many other cancer types. This higher level of protein content observed could be explained by as previously suggested, it functioning as a more prominent driver of exosome mode of action.

Hierarchical clustering showed that within the exosome samples was a clear separation of target expression between normal and diagnostic with post-BMT samples. In terms of the type of dominant protein species, the diagnostic and normal samples showed a dominance of adherent related targets compared to a dominance of immunology based functions in early BMT changing to a survival/differentiation profile in mid-late BMT.

The leukaemic environment in AML is highly immunosuppressive and early post-transplant it would not be unexpected to see high expression of targets associated with immune response as the patient would still be in an immune suppressed state, the change in expression profile to one of survival/differentiation in some mid-late BMT samples shows that the patients are recovering a more supportive environment for haematopoietic growth. It is unexpected to not see higher levels of immune related targets within the diagnostic samples as previously mentioned, this would also be an immunosuppressed environment, a possible explanation for this might be that the malignant environment has already undergone changes to compensate for this and so less transfer of information via exosomes is required, unlike that seen in post-transplant which is undergoing much change within the microenvironment alongside the effect of immunosuppressants requiring more intercellular communication. One of the targets I identified within the post-BMT samples as highly expressed is IL-12 which has been associated with the activation of NK-cells and increased anti-leukaemic activity, it has also been suggested that IL-12 activated NK-cells help prevent GvHD taking place by inhibiting the proliferation of donor T-cells while still maintaining a GvL effect, improving the OS of mice (Ewen et al. 2018).

Although both VCAM-1 and ICAM-1 were identified as being highly expressed within all stages of patient exosome samples, this is not unexpected as all cell types within the bone marrow microenvironment require high levels of adherence in order to retain healthy or diseased cells and create the associated hospitable niche. The expression of these targets was observed to be higher within the diagnostic samples compared to NBM. This was particularly apparent within the validation western blots that showed

a complete absence of ICAM-1 within the two normal exosome samples in contrast to a clear upregulation within the diagnostic and post-BMT samples suggesting significant remodelling and potential for adhesion to the niche in these samples. Other studies have showed similar results, work carried out using breast cancer cell lines showed that cancer derived exosomes associated with the protein fetuin-A and histone H2A which resulting in exosome mediated adhesion and cell spreading (Nangami et al. 2014). Work involving mature dendritic cells showed that secreted exosomes were enriched in ICAM-1 functioning as important adhesion receptors which resulted in the efficient activation of T-cells (Segura et al. 2005). Exosomes are reported to play a major role in transforming the AML BM-ME into more hypoxic conditions, which can result in the upregulation of TGF-β1 expression and subsequent AML blast proliferation. This exosomal driven chain of events can lead to activation of CXCR4/SDF-1 pathway, immune cell suppression and increased levels of VCAM-1 and ICAM-1 such as I observed which aid regulation of cell adhesion and remodelling of the BM-ME (Chen et al. 2013; Ohyashiki et al. 2016).

An increase in ICAM-1 within the post-BMT samples could also be explained by the role it plays in bringing about an immune response. Previous studies have shown that B-cells actively uptake exosomes and interact with those exosomes containing MHC-class II and ICAM-1 originating from mature dendritic cells. Leukocyte function associated antigen-1 (LFA-1) is used by activated T-cells for binding the DC exosomes and it is the interaction between LFA-1 and ICAM-1 which is critical for priming naïve T-cells and the subsequent antigen specific immune response triggered (Ferguson Bennit et al. 2019). Future work could involve investigating the presence of these activated cell types within the bone marrow to monitor changes in the active T-cell population. This could be accomplished by monitoring the presence of T-cell associated molecules like LFA-1 and MHC within serial AML patient plasma samples which we have banked in storage. Elevated levels of ICAM-1 within AML has been reported in previous work, ICAM-1 commonly present on leukaemic blasts leads to increased adhesion of these cells within the BM niche leading to a series of events which include protection, quiescence and chemoresistance which is commonly followed up by AML blast detachment, increased proliferation and relapse (Gruszka et al. 2019). The adhesion properties supplied by ICAM-1 and other related molecules give rise to an acquired therapeutic resistance and could possibly be used as a good indicator of disease relapse.

TIMP-1 (Tissue inhibitor of metalloproteinase 1) was identified by Luminex and ELISA as having higher levels within the post-BMT exosome samples, recent studies have

shown it to have a crucial role in immune regulation as well as links to inflammatory and autoimmune disorders which could explain it's increased prevalence in post-BMT. Interestingly, a recent study investigated potential biomarkers for GvHD in post-alloHSC transplant patients using plasma from a variety of haematological disorders, within a large cohort they identified only TIMP-1 as a viable potential biomarker. A correlation between TIMP-1 and GvHD risk was identified and showed that patients with high TIMP-1 levels were twice as likely to experience GvHD compared to those patients with normal levels (Shin et al. 2019). Monitoring GvHD is an important way of also assessing the anti-leukaemic effect of GvL as previous work has shown that the T-cells of the marrow graft are responsible for both initiating GvHD and enhancing GvL effect (Horowitz et al. 1990). The prevention of GvHD while enhancing the beneficial aspects of GvL is the most effective way of reducing the chance of leukaemic relapse after bone marrow transplant (Tsukada et al. 1999).

Studies have also shown an increased level of TIMP-1 within BM plasma of AML patients compared to normal, they demonstrated that TIMP-1 is involved in modulating leukaemic blast migration and survival (Forte et al. 2017). Another study showed that exosomes derived from MSCs contained elevated levels of TIMP-1 and supported tumour function when introduced into a xenograft assay involving MCF7 breast cancer cells. Once again, the observations shown in the exosome fraction were not reflected within the supernatant.

The difference between exosome and supernatant fractions within the analysis of target abundance isn't large within the post-BMT subset both show an increase in levels of immunology related targets at early stage changing at late stage to become more survival/differentiation related. When analysing specific targets a more defined difference between exosome and supernatant becomes apparent especially within those immunology related targets; TIMP-1, ERBB2, IL-12 and IL-4.The cargo of exosomes is known to contain information that differs to that of the parental cell (Hornick et al. 2015) and is thought to be directed and specifically targeted to acceptor cells in order to bring about a phenotypic change (Ciardiello et al. 2016). Supernatant fractions consist of a very heterogenous mix of metabolites, growth factors and matrix proteins which although give useful information regarding the microenvironment of the residing cells this doesn't provide details of the targeted and specific changes being induced as shown by the exosome contents. There is strong evidence showing the strong efficacy MSC secreted factors have in mediating tissue repair and regeneration via paracrine methods (Togel et al. 2005; Togel et al. 2007; Xiang et al. 2009; Park et al. 2012), but the mechanisms are not fully understood and it is known

that EVs make up a large portion of the MSC secretions and these effects have been shown to have equal potency to those of whole cell MSC administration (Rani et al. 2015), meaning that the functional effects could in theory be predominantly due to the MSC EV secretions. The exosome results provide a more informative overview of intercellular communications and potential functional output and more specifically show an increased association with immunomodulatory function, which could become an early indication of relapse and signifies the importance of changes observed between samples of different origin; normal, diseased or post-transplant.

Previous studies have shown that MSCs release several soluble factors linked to immune suppressive and anti-inflammatory properties these include extracellular vesicles which have also been suggested as a potential therapeutic in supporting stem cell transplant (Uccelli et al. 2008). Data showed that the advantages of exosomes over MSCs are the unlikelihood of immune rejection due to their small size and lower expression of histocompatibility molecules, they have also shown to reduce or stop GvHD by modulating the immune response and this in combination with HSCs could help create a more pro-haematopoietic BM-niche (Reis et al. 2016).

Luminex analysis highlighted higher levels of ERBB2/HER-2 within the post-BMT and diagnostic exosome samples. ERBB2/HER2 is a tyrosine kinase whose overexpression is seen in many cancers most commonly as a breast cancer oncogene, but also implicated in LSC maintenance and promotion of leukaemogenesis (Ufkin et al. 2014). It is known to bring about cellular affects via activation of the PI3 Kinase/AKT pathway of which downstream effects can include increased expression of ERK and promotion of PD-L1 mediated immune response (Hong et al. 2016).

Within a stromal co-culture setting I showed an increase in ERK expression within exosome fractions, this along with the overexpression of ERBB2 seen in diagnostic and post-BMT exosomes suggests a potential role for exosomes in conferring this dysregulation. It is interesting to observe that the increased levels of ERBB2 is not reflected in the supernatant fraction of Luminex analysis, suggesting that mobilisation of this molecule and subsequent signalling transduction is due to exosome transfer alone. The mediation of intercellular transfer of molecules by extracellular vesicles has been identified in numerous studies especially within the PI3K/Akt signalling pathway, but as yet not within the post-BMT setting of AML (Gangoda et al. 2015). When exosomes were introduced into a stromal co-culture, I observed an overall increase in leukaemic cell proliferation along with an activation of ERK. It is widely

reported that when myeloid blasts are in contact with stroma many pathways are upregulated including ERK (Roccaro et al. 2013; Viola et al. 2016), it has also been shown that leukaemic blasts on stroma have influenced the inhibition of FLT3 via a combination of soluble factors and direct contact with stromal cells (Parmar et al. 2011; Yang et al. 2014).

A previous study investigated inhibitory signals thought to be initiated by blast derived exosomes which when co-incubated with NK cells caused a down regulation of NKG2D suppressing their function. A mixed population of exosomes were separated using CD34+ antibody coated magnetic beads and contents of the different populations analysed. They found that the CD34+ blast derived exosomes contained content which resembled that of parental blasts, and the enrichment correlated directly with the % of blasts in circulation which was not seen in the normal derived exosomes, confirming the hypothesis that leukaemic blasts control their exosomal cargo and it differs to that of a normal derived exosome (Hong et al. 2014a). This technique has been criticised as drawbacks include the difficulty identifying an appropriate surface target and antibody which recognises the extracellular domain, it's also possible that due to the heterogeneity of diseased cells that not all will express the target antigen and with disease progression this could be lost or the antigenic epitope could become masked or blocked leading to low specificity (Taylor and Shah 2015).

Separating exosomes would have been helpful within my studies enabling me to identify the exact origin of my mixed exosome preparations, this was a limitation and going forward pinpointing the origins of specific exosomal populations and their individual functional input within a mixed system, would be a useful analytical tool. I showed an activation of ERK in exosomes when on stroma although it is unknown whether HS5 or leukaemic cell derived exosomes are driving this signal upregulation, this observation warrants further investigation and separating exosome populations to enable the identification of the origin of the ERK activated exosomes would help gain insight into this mechanism of action.

In order to address the further mechanisms of stromal and exosome support a future work plan will include a study of these interactions using a 3D cell culture model which will give us a simplified model of the bone marrow niche allowing us to assess the interactions between cell types within an *ex vivo* setting, providing an important element of vasculature and providing a closer study of defined cell interactions and produce functional readouts of cell to cell communication such as movement,

adhesion and migration. Experiments could also be carried out under normoxic and hypoxic conditions to assess the effect this has on exosome release as this is known to be an important factor within the malignant bone marrow microenvironment. This model could also be used to differentiate between transient and permanent reprogramming taking place due to the ability to switch 'on and off' *in vitro* experiments. The fluorescent labelling of exosomes to track their movement could also be incorporated and assessed using confocal microscopy, this was recently used in a study investigating the chemoresistant effect created by the AML blast exosome instigation of IL8 release from BM stem cells (Chen et al. 2019). To current knowledge this methodology has not been used within the context of post-BMT AML and would allow closer study of defined cell interactions.

In summary, AML is a heterogeneous disease which is still fatal for the majority of patients within high risk category, it is unlikely that any pharmacological drug treatment will resolve the issue of residual disease contributing to relapse therefore it is imperative that other strategies are developed to target both pre and post BMT and improve treatment. Within this thesis I have successfully isolated and characterised extracellular vesicles from normal and pre and post BMT AML patients and shown that within a diseased or recovering BM-ME EVs are present in higher levels compared to healthy individuals indicating their involvement in promoting malignancy. Identification of EV content showed a clear predisposition for immunomodulation function post- transplant and an associated miRNA and mIR expression within this patient sub-set signified the heterogeneous nature and potential indicative impact it could have on therapeutic options. These observations warrant further exploration as they could improve understanding of the mechanisms involved in BM remodelling within the leukaemic niche and potentially provide an early indicator of relapse.

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