An ex vivo investigation of interactions between primary acute myeloid leukaemia and mesenchymal stromal cells yields novel therapeutic targets

Acute myeloid leukaemia (AML) relapse rates are high, at 60–80% within three years of diagnosis. This may be due to sequestration of chemoresistant leukaemic stem cells within the bone marrow (BM) microenvironment. Mesenchymal stromal cells (MSCs) are the common progenitor for BM stroma, and can differentiate into cells of mesodermal origin. Previous reports have shown that mesenchymal stromal cell (MSC) modification without functional alteration of the haematopoietic system causes subsequent myelodysplasia and secondary leukaemia in murine models. In this study, we characterise the impact of primary patient-derived AML cells upon MSC populations in an ex vivo setting. Our findings identify key differentially expressed genes across the MSC transcriptome in the context of AML, and compare the difference in gene expression between normal and cancer phenotypes. We identify both well-established and novel genes. The latter may confer growth benefits by upregulating canonical cancer pathways in AML cells, such as Wnt/β-catenin (as in the case of secreted frizzled related protein 4, SFRP4), or altering immune and metabolic pathways (such as retinoic acid receptor responder 1, RARRES1). These may constitute novel targets for therapeutic interventions targeting the BM microenvironment.

MSC samples from a normal paediatric donor, provided by the John Goldman Centre for Cellular Therapy (Hammersmith Hospital, London) were immunophenotyped by staining with the standard reference panel of labelled mouse anti-human antibodies (CD105-APC, CD73-PE, CD90-APC, CD45-FITC, CD34-PE, CD3-APC, CD19-PE, HLA-DR-FITC, anti-human antibodies (CD105-APC, CD73-PE, CD90-APC, CD45-FITC, CD34-PE, CD3-APC, CD19-PE, HLA-DR-FITC and CD14-PE, BD Biosciences, Franklin Lakes, NJ, USA) as defined by the International Society of Cellular Therapy using a FACS Canto II analyser (BD Biosciences) on FACS Diva software. These were plated into the lower compartments of a 24-well plate with 0·4 μm polycarbonate Transwell inserts (Corning, Cornning, NY, USA) and incubated for 24 h. At 24 h, AML blasts or normal cells were plated into the upper compartment of each well. In total, triplicate co-cultures were set up for three individual AML blast donors (characteristics shown in Table I) and one normal CD34+ donor, with the remaining 12 wells used as a negative control (upper compartment empty). After 72 h of coculture, RNA was extracted from MSCs using the RNeasy Mini kit (QIAGEN, Hilden, Germany) per the manufacturer’s instructions and paired-end sequencing undertaken on the HiSeq 4000 system (Illumina, San Diego, CA, USA). Differential gene expression analysis was then performed (see Figure S1 for details).

Overall, analysis yielded 57 differentially expressed genes in MSCs when comparing the transcript numbers in AML MSCs against non patient (NP) MSCs: 26 protein-coding genes, 8 processed pseudogenes, 12 micro-RNAs, 4 small nucleolar RNAs, 4 long non-coding RNAs and 3 miscellaneous RNAs (Table II). Forty-seven of these were upregulated in AML MSCs, and 10 were downregulated.

Table I. Patient age at diagnosis and associated morphological, cytogenetic and molecular characteristics of AML blast cells.

| Case ID | Age at diagnosis | AML type     | Morphological classification | Cytogenetic findings                  | Molecular findings              |
|---------|-----------------|--------------|-----------------------------|--------------------------------------|---------------------------------|
| AML 14  | 67              | De novo      | Acute monoblastic leukaemia (FAB M5) | 46,XX,tt(16;16)(p13;q22)[4]/46,XX[14] | -                               |
| AML 15  | 24              | De novo      | AML without maturation (FAB M1) | 46,XX,del(13)(q13q21)[3]/46,XX[17]   | FLT3/ITD                        |
| AML 16  | 52              | De novo      | AML without maturation (FAB M1) | 46,XY[20]                            | FLT3/ITD (two tandem duplications) and NPM1 mutations |

constant spring disease.
Table II. Differentially expressed (a) protein-coding and (b) non-coding genes with gene biotype at \( q = 0.8 \), expressed as log₂-fold change. For protein-coding genes, this also includes functional domains as reported in the literature.

| Gene name | Log₂-fold change | Reported functions | Membrane or secreted proteins | Gene name | Biotype | Log₂-fold change |
|-----------|------------------|--------------------|-------------------------------|-----------|---------|------------------|
| SLC7A2    | 4.50             | I, M               | Membrane                      | SEC62-AS1 | IncRNA  | 4.97             |
| CXCL8     | 4.25             | E, G, I            | Secreted                      | HSPLA8    | Processed pseudogene | 4.65 |
| CXCL1     | 4.03             | E, G, I            | Secreted                      | RNU6-33   | Processed pseudogene | 4.39 |
| LIPM      | 3.98             | M                  | Secreted                      | NREP      | Processed pseudogene | 4.21 |
| CXCL5     | 3.97             | E, G, I            | Secreted                      | MIR548A3  | miRNA   | 4.19             |
| CXCL6     | 3.70             | E, G, I            | Secreted                      | NPY       | Y RNA    | 4.03             |
| MMP9      | 3.62             | E, G, I            | Secreted                      | SNORD114-11 | snoRNA | 3.75             |
| MMP18     | 3.36             | E, G, I            | Secreted                      | RPLP1     | Processed pseudogene | 3.65 |
| CXCL3     | 2.78             | E, G, I            | Secreted                      | SNOU13    | snoRNA   | 3.63             |
| POU2P2    | 2.70             | E, I               | Secreted                      | MIR3138   | miRNA   | 3.62             |
| CXCL2     | 2.70             | C, E, G, I         | Secreted                      | RNU6-302P | Processed pseudogene | 3.42 |
| NTN1      | 2.67             | E, G               | Secreted                      | RNU6-1024P | Processed pseudogene | 3.41 |
| ADAP1     | 2.64             | E                  | Secreted                      | SNORD113  | snoRNA   | 3.27             |
| TRPA1     | 2.54             | E, I               | Membrane                      | MIR4451   | miRNA   | 3.20             |
| CLDN1     | 2.27             | E, G               | Secreted                      | MIR199A1  | miRNA   | 3.08             |
| HSD11B1   | 2.18             | G, I, M            | Secreted                      | MIR4324   | miRNA   | 3.06             |
| IL6       | 1.93             | C, E, G, I         | Secreted                      | ACTL6A    | IncRNA  | 3.06             |
| BEX1      | 1.88             | G                  | Secreted                      | COP5      | IncRNA  | 2.81             |
| AB13BP    | 1.83             | G                  | Secreted                      | MIR6894   | miRNA   | 2.58             |
| MMP13     | 1.75             | E, G, I            | Secreted                      | MIR6784   | miRNA   | 2.38             |
| NR4A2     | 1.74             | E, G, I            | Secreted                      | MIR6865   | miRNA   | 2.38             |
| TNFAIP6   | 1.66             | G, I               | Secreted                      | MIR6090   | miRNA   | 2.31             |
| ZC3H12A   | 1.50             | G                  | Secreted                      | MIR4284   | miRNA   | 1.82             |
| RARRES1   | −1.54            | E, G, I, M         | Secreted                      | LNC-DHRS3-1 | IncRNA | −1.68            |
| SFRP4     | −1.58            | E, G, I            | Secreted                      | RNU6ATAC18P | Processed pseudogene | −1.88 |
| KRT18     | −1.99            | E                  | Secreted                      | RNA55P383 | rRNA pseudogene  | −1.93 |
|           |                  |                    |                               | MIR4730   | miRNA   | −2.00            |
|           |                  |                    |                               | RNU7-40P  | Processed pseudogene | −2.15 |
|           |                  |                    |                               | MT-TM     | miRNA   | −2.75            |
|           |                  |                    |                               | MIR542    | miRNA   | −3.16            |

C, chemoresistance; E, extracellular matrix breakdown and invasion; G, pro-growth and anti-apoptosis; I, pro-inflammation and immunomodulatory; M, altered metabolism.
Differentially expressed protein-coding genes could broadly be categorised according to their function in the context of cancer, with many being previously identified in either AML or in solid tumours (Table IIa). In total, 20 were reported to be pro-growth or anti-apoptosis; 19 were involved in extracellular matrix breakdown or tumour invasion; 2 conferred chemoresistance; 19 had inflammatory and immunomodulatory functions; and 4 altered cellular metabolism. Some genes fell into multiple categories. Furthermore, 15 of the proteins coded by these genes are secreted by cells, and 3 are membrane proteins.

Over half of the identified differentially expressed genes were non-coding elements. These have been reported as having complex expression profiles and putative effects in the context of cancer.6

We identified several MSC genes that are well established in the literature as beneficial to AML, such as the CXCL family, suggesting our study methods were valid. For example, CXCL8, a pro-inflammatory cytokine with neutrophil attractant properties which activates several canonical cancer pathways including MAPK and PI3K,7 was upregulated in our study [4.25 log2-fold change; (LFC)].

Two genes of potential interest identified in this study were SFRP4 and RARRES1, both of which were downregulated in AML MSCs (−1.15 and −1.54 LFC respectively). SFRP4 is a secreted protein which acts by inhibiting the Wnt/β-catenin pathway.8 Enhanced activity of Wnt/β-catenin has been shown in both AML cell lines and primary blasts,9 and SFRP4 downregulation predicts poor prognosis in AML subtypes.10 One of the mechanisms for this may be through decreased SFRP4 secretion by MSCs.

MAPK/mTOR pathway activity is enhanced in AML.11 This study found downregulation of RARRES1, a known MAPK/mTOR pathway repressor and autophagy inducer.12 RARRES1 has also been associated with metabolic shifts towards de novo lipogenesis in AML13 and in vivo alterations of haematopoietic progenitor cell (HPC) proliferation and differentiation.14 Since RARRES1 is not a secreted protein, it is unlikely to directly reduce mTOR pathway activity in AML cells; however, these pleiotropic effects may be a result of alterations in other secreted proteins. This warrants further investigation.

MSCs for experiments were sourced from a paediatric donor, as paediatric cells may have greater growth potential and resilience than those from older donors. However, there remains a concern that the expression profiles obtained may differ based on donor age.

Although this exploratory study used primary patient-derived cells to maximise similarity to clinical phenotypes, small sample sizes limited the available analyses. This was partially mitigated using NOISeq, an R package for differential gene expression analysis which has been demonstrated to have a lower false discovery rate at sample sizes of three or fewer than other alternatives.15 Unfortunately, due to the Covid-19 pandemic, we were unable to mechanistically validate identified genes.

In conclusion, this study utilised a well-established co-culture model to investigate transcriptome-wide gene expression alterations in BM MSCs in the context of AML using primary patient-derived cells. Several differentially expressed genes had pleiotropic effects, conferring benefits upon AML cells through diverse mechanisms. Although mechanistic studies will be required to validate and expand upon these findings, this study provides an initial basis from which to further characterise AML–BM interactions. In particular, we identify SFRP4 and RARRES1 as potential novel routes through which MSC modification may augment AML cell survival.

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**Author contributions**

AC performed the experiments, analysed the data and wrote the manuscript; SL and EYF provided research material and reviewed the manuscript; ENM and JFA advised on the clinical aspect of the project and reviewed the manuscript; JSK designed the experiments, supervised the project and reviewed the manuscript.

**Conflict of interest**

The authors have no conflicts of interest to declare.

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Severe macrothrombocytopenia with platelet CD9 deficiency responsive to romiplostim

The spectrum of inherited platelet disorders with variable platelet size continues to expand.1,2 Among them, macrothrombocytopenia cases constitute a subgroup with different genetic basis, and mild-to-moderate thrombocytopenia without major bleeding symptoms. Here we describe a child with symptomatic severe macrothrombocytopenia without a genetic aetiology and low platelet CD9 expression raising the possibility of a new form of inherited macrothrombocytopenia.

A 6-month-old female presented with diffuse ecchymotic skin lesions, severe thrombocytopenia (<10 × 10^9/l) and giant platelets Fig 1A–D. She has normal growth and development without any dysmorphic features. She was unresponsive to intravenous immunoglobulin and steroid treatments. Bone marrow was normocellular with normal megakaryocyte density and morphology, and cytogenetic evaluation. Family history revealed that the parents were first-degree cousins, originally from Yemen; however, two sisters and the parents had no history of bleeding with normal platelet counts.

Genetic studies for bone marrow failure and macrothrombocytopenia including glycoprotein Ibα (GP1BA), glycoprotein Ibβ (GP1BB), glycoprotein IX (GP9) by Sanger sequencing revealed no variants. The patient was given weekly platelet transfusions leading to transient count recovery and resolution of the bleeding symptoms Fig 1E. Platelet flow cytometry analysis and ristocetin-induced aggregation studies did not show any abnormalities, as the tested platelets were the transfused cells.

With no underlying cause for macrothrombocytopenia and continued dependence on platelet transfusions, the patient was started on thrombopoietin (TPO) receptor agonist (RA), eltrombopag after reviewing the potential side-effects with parents. Ertrombopag formulation production was discontinued by the manufacturer, therefore she began treatment with another TPO-RA, romiplostim, which is administered subcutaneously on a weekly schedule providing platelet counts of >100 × 10^9/l with resolution of bleeding symptoms Fig 2A. The patient has been maintained on romiplostim injections for 9 months with continued giant platelets on the periphery.

Whole exome analysis by next generation sequencing did not reveal a known variant, but heterozygous mutation in ankyrin repeat domain 26 (ANKRD26) and homozygous mutation in RNA component of mitochondrial RNA processing endoribonuclease (RMRP) with unknown