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BRIEF REPORT

REVISED A human mesenchymal spheroid prototype to replace moderate severity animal procedures in leukaemia drug testing [version 2; peer review: 1 approved, 1 approved with reservations]

Aaron Wilson[1*], Sean Hockney[2*], Jessica Parker[2], Sharon Angel[1], Helen Blair[1], Deepali Pal ID [1,2]

[1]Wolfson Childhood Cancer Research Centre, Translational and Clinical Research Institute, Newcastle University, UK, Newcastle upon Tyne, NE1 7RU, UK
[2]Applied Sciences, Northumbria University, Newcastle upon Tyne, NE1 8ST, UK

* Equal contributors

Abstract
Patient derived xenograft (PDX) models are regarded as gold standard preclinical models in leukaemia research, especially in testing new drug combinations where typically 45-50 mice are used per assay. 9000 animal experiments are performed annually in the UK in leukaemia research with these expensive procedures being classed as moderate severity, meaning they cause significant pain, suffering and visible distress to animal's state. Furthermore, not all clinical leukaemia samples engraft and when they do data turnaround time can be between 6-12 months. Heavy dependence on animal models is because clinical leukaemia samples do not proliferate *in vitro.* Alternative cell line models though popular for drug testing are not biomimetic – they are not dependent on the microenvironment for survival, growth and treatment response and being derived from relapse samples they do not capture the molecular complexity observed at disease presentation. Here we have developed an *in vitro* platform to rapidly establish co-cultures of patient-derived leukaemia cells with 3D bone marrow mesenchyme spheroids, BM-MSC-spheroids. We optimise protocols for developing MSC-spheroid leukaemia co-culture using clinical samples and deliver drug response data within a week. Using three patient samples representing distinct cytogenetics we show that patient-derived-leukaemia cells show

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	1	2
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1. **Simon E. Richardson** ID, Wellcome - MRC Cambridge Stem Cell Institute, Cambridge, UK

2. **Helen Wheadon**, University of Glasgow, Glasgow, UK

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enhanced proliferation when co-cultured with MSC-spheroids. In addition, MSC-spheroids provided improved protection against treatment. This makes our spheroids suitable to model treatment resistance – a major hurdle in current day cancer management

Given this 3Rs approach is 12 months faster (in delivering clinical data), is a human cell-based biomimetic model and uses 45-50 fewer animals/drug-response assay the anticipated target end-users would include academia and pharmaceutical industry. This animal replacement prototype would facilitate clinically translatable research to be performed with greater ethical, social and financial sustainability.

Keywords
Animal replacement, 3D models, Preclinical models, cancer research, leukaemia

This article is included in the NC3Rs gateway.

Corresponding author: Deepali Pal (deepali.pal@northumbria.ac.uk)

Author roles: Wilson A: Investigation, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Hockney S**: Investigation, Visualization, Writing – Review & Editing; **Parker J**: Investigation, Visualization, Writing – Review & Editing; **Angel S**: Investigation, Visualization, Writing – Review & Editing; **Blair H**: Investigation, Methodology, Project Administration, Resources, Supervision, Visualization, Writing – Review & Editing; **Pal D**: Conceptualization, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Supervision, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing

REVISED **Amendments from Version 1**

This version addresses key points raised by the reviewers and includes additional data. We have clarified throughout the text, including in the abstract and introduction, that the disease type described here refers to acute lymphoblastic leukaemia. We have also replaced the term "blast" with ALL cells. As per reviewer suggestion we have now included additional data. These include analysis of CD105, CD90 and CD73 expression in 2D versus 3D MSC to validate 3D MSC via expression of key markers at protein level, Hoechst-33342 and calcein staining of BM-MSC spheroids as live cell analysis of the BM-MSC spheroids, analysis of ALL cell death following dexamethasone treatment, drug combination matrix analysis in 2D versus 3D (additional data) versus *in vivo* (from mouse experiments that were performed in previous studies) format to gain insight into the capacity of the 2D and 3D *in vitro* models towards *in vivo* predictivity, and drug response testing on BM-MSC to rule out killing of BM-MSC by the drugs. All additional experiments have been accompanied by revised text in results, discussion and the methods section as well as revised figures throughout. Furthermore, an additional section, "Study Limitations", has been included to discuss the restrictions and scope of this brief report. All new data has been included in the Mendeley data repository. Authorship list and order has remained unchanged, however in view of additional experiments, Sean Hockney has now been included as joint first author along with Aaron Wilson.

Any further responses from the reviewers can be found at the end of the article

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Research highlights

Scientific Benefit

A 3D spheroid-based approach with improved clinical relevance for *ex vivo* co-culture of patient-derived-leukaemia samples.

3Rs Benefit

To replace animal experiments, including moderate severity animal (mice) procedures in leukaemia research and drug testing.

Practical Benefit

A 3Rs approach that yields drug response data quickly and is ethically, socially and financially more sustainable than its *in vivo* counterparts.

Current applications

Exploration of leukaemia biology such as stem/progenitor proliferation, stem/progenitor-niche interactions, niche-impacted treatment resistance and to obtain drug response data.

Potential applications

Adapt the approach to include other haematological cancers as well as bone cancers.

Introduction

Acute lymphoblastic leukaemia (ALL) management in children is hindered by two chief obstacles: 1. Treatment toxicity[1,2] 2. 15-20% of patients go into relapse, when the disease can become treatment resistant.[3] This highlights the requirement for improved treatments. High drug attrition rates remain a major hindrance to anti-cancer drug development. 95% of de novo drugs entering phase 1 trials never reach patients.[4–6] Three key improvements[5] have been identified to reduce attrition rates: 1. Increase in scientific collaborations 2. Tumour biology driven preclinical studies and clinical trials 3. Improved development of translatable preclinical cancer models that would screen out "false positives" therapeutics before these reach clinical trials.[5]

The gold standard preclinical model in leukaemia research is a PDX model.[7–9] Recent Home Office annual returns show that 195,407 mice are used annually in cancer research. Indeed, cancer research remains the most common applied sciences area to use animal procedures. Given leukaemia research is heavily dependent on mouse models combining these metrics with financial data from major cancer funders in the UK suggest that 5% of these mice or at least 9000 mice are used in leukaemia research every year. Although leukaemia is a very aggressive disease, leukaemia cells do not maintain viability outside the body.[9] Several research groups would likely use primary patient for *in vitro* studies before moving into *in vivo* models, nevertheless *in vivo* models are still used as key models to expand primary samples as vital research resources. This causes heavy reliance on PDX models, where leukaemia cells are engrafted into immunocompromised mice to amplify patient-derived leukaemia cells, to study cancer biology and perform translational studies.[7,8] Patient-derived leukaemia cells can take 2-12 months to engraft in mice before any preclinical drug testing can begin. Furthermore, most of these procedures involve mice developing significant adverse effects such as weight loss, fur ruffling, back hunching as well as increased white cell counts.

The pipeline for preclinical testing using mouse PDX models is: 1. Toxicity analysis to determine no observed adverse effect limit (NOAEL)/maximum tolerated dose (MTD): 15-20 mice per drug.[9] 2. Pharmacokinetic studies to determine drug maximum plasma/tissue levels and kinetics of drug clearance:15-30 mice per drug.[10] 3. Drug efficacy studies and pharmacodynamics assays on tumour cell response: 5-10 mice needed per drug treatment group.[11] Per drug group a total of 40-50 animals are needed for single drug assays and twice as many for drug combination assays.[9–11] Moreover, *in vitro* cancer models preceding *in vivo* validation experiments rely on cell lines, which retain cytogenic characteristics of leukaemia. However being derived from patients with relapsed disease these samples do not represent the molecular complexity of disease that is observed at presentation.[12,13] Cell lines have furthermore adapted to grow *in vitro* as suspension monocultures, and therefore do not represent cancer within the context of its microenvironment. Therefore, these models show limited *in vivo* predictivity, leading to increase *in vitro* to *in vivo* drug attrition rates.

ALL is a disease primarily of the bone marrow (BM), the BM comprises cell types including haematopoietic, mesenchymal stroma cells (MSC), osteoblasts, osteoclasts, endothelia, perivascular and immune cells. Difficulty in culturing patient derived ALL cells *ex vivo* alludes to the vital role that the BM-niche plays in cancer biology. The leukaemia BM-microenvironment plays a key role in disease, mediating cell-cell signalling and conferring treatment protection to leukaemia cells.[14,15] Mesenchyme-leukaemia interactions in the BM generate two leukaemia subpopulations, slow cycling, dormant- and actively cycling, disease propagating- populations.[12,16] Many treatments are effective in reducing active cycling leukaemia cells; however, dormant leukaemia-populations can survive treatment, seeking refuge within the BM-microenvironment. These dormant cells can later, post treatment, shift back into a cycling state, leading to disease relapse[13,16] and therefore capturing dormant ALL cells *in vitro* is important. Furthermore, targeting leukaemia-niche interactions may present as promising therapeutic approaches. We have developed *in vitro* 2D co-culture platforms in previous studies[12,17] to 1. conduct preclinical drug screening using patient-derived leukaemia cells, thereby reducing dependence on cell lines, consequently minimising *in vitro* to *in vivo* drug attrition rates and 2. explore the impact of human BM cells in driving ALL biology such as leukaemia proliferation, dormancy and treatment resistance.[12] Furthermore, to build proof-of-confidence in application data, we previously showed that such human relevant *ex vivo* platforms have potential to detect therapeutically exploitable targets that disrupt leukaemia-BM cell interactions conferring treatment resistance to the cancer cells.[12,18] Such models also have greater tractability than *in vivo* procedures, further mitigating reliance on mouse experiments to perform mechanistic studies. However, standard 2D models, including co-cultures, do not sufficiently portray functional biomimicry and do not recapitulate structural intricacies of the leukemic niche. Nor do such 2D models capture the complex polarity of cell-cell interactions[19] that are likely to influence leukaemia cell growth.

Recent advances in medicine have embraced 3D models as miniature *in vitro* representations of specific organs.[20] 3D models have been proven to be useful models of human disease, particularly in cancer research.[21] Modern medicine is embedding the use of organ-on-a-chip systems which have led to advances in liver and lung drug screening, providing more accurate toxicological reports than 2D systems.[20] The complex and dynamic nature of ALL and the BM mesenchymal microenvironment highlights the need for a more representative drug screening platform. This paper aims to do this by developing mesenchymal spheroids, to support the co-culture of patient derived leukaemia samples, consequently enabling stratified-medicine and precision oncology driven therapeutic investigations.[22]

Methods and materials
Materials

Table 1. Materials used.

Material	Manufacturer	Catalogue Identifier
General		
Trypsin-EDTA Solution 10X	Sigma-Aldrich, Dorset, UK	59418C
Phosphate-Buffered Saline	Thermo Fisher Scientific, Hertfordshire, UK	10010023
Gentle Cell Dissociation Reagent	Stem Cell Technologies, Stem Cell, UK	**100-0485**
Heat Inactivated Foetal Bovine Serum	Thermo Fisher Scientific, Hertfordshire, UK	10500064
Matrigel HESC-Qualified Matrix	Corning, Kennebunk, USA	CLS354277
Low-glucose Dulbecco's Modified Eagle's Medium	Sigma-Aldrich, Dorset, UK	D5546

Table 1. *Continued*

Material	Manufacturer	Catalogue Identifier
Cells		
Primary Mesenchymal Stem Cells	Obtained from patients undergoing total hip replacement in view of osteoarthritis[17]	
PDX sample L707	Obtained from patient with E2A-HLF translocation[12,17]	
PDX sample L49120	Obtained from patient with BCR-ABL translocation[23]	
PDX sample MS40	Obtained from patient with MLL rearrangement[24]	
MSC culture media		
Low-glucose Dulbecco's Modified Eagle's Medium	Sigma-Aldrich, Dorset, UK	D5546
Heat Inactivated Foetal Bovine Serum	Thermo Fisher Scientific, Hertfordshire, UK	10500064
Penicillin/Streptomycin	Sigma-Aldrich, Dorset, UK	P4333
Basic FGF	Sigma-Aldrich, Dorset, UK	PHG0024
L-Glutamine	Sigma-Aldrich, Dorset, UK	59202C
Matrigel HESC-Qualified Matrix	Corning, Kennebunk, USA	CLS354277
Leukaemia Blast Co-Culture		
SFEM, Serum-Free Medium for Culture and Expansion	Stem Cell Technologies, Stem Cell, UK	09650
Organoid formation		
AggreWellM 400 24-well Plate	Stem Cell Technologies, Cambridge, UK	34411
AggreWell Rinsing Solution	Stem Cell Technologies, Cambridge, UK	**07010**
Costar Ultra-low Attachment 6 Well Plate	Corning, Kennebunk, USA	3471
Real-Time PCR Analysis		
QIAShredder	Qiagen, Manchester, UK	79656
Beta-mercaptoethanol	Sigma-Aldrich, Dorset, UK	M6250
RNEasy Mini Kit	Qiagen, Manchester, UK	74104
RNase-Free DNase Set	Qiagen, Manchester, UK	79254
Primer Sequences		
CDH2 forward primer, Sigma-Aldrich, Dorset, UK	GGTGGAGGAGAAGAAGACCAG	OLIGO
CDH2 reverse primer, Sigma-Aldrich, Dorset, UK	GGCATCAGGCTCCACAGT	OLIGO
CD90 forward primer, Sigma-Aldrich, Dorset, UK	CACACATACCGCTCCCGAAC C	OLIGO
CD90 reverse primer, Sigma-Aldrich, Dorset, UK	GCTGATGCCCTCACACTT	OLIGO
NES forward primer, Sigma-Aldrich, Dorset, UK	AGAGGGGAATTCCTGGAG	OLIGO
NES reverse primer, Sigma-Aldrich, Dorset, UK	CTGAGGACCAGGACTCTCTA	OLIGO
GAPDH forward primer, Sigma-Aldrich, Dorset, UK	GAAGGTGAAGGTCGGAGTC	OLIGO
GAPDH reverse primer, Sigma-Aldrich, Dorset, UK	GAAGATGGTGATGGGATTTC	OLIGO

Table 1. *Continued*

Material	Manufacturer	Catalogue Identifier
Staining Reagents		
Calcein AM	BioLegend	425201
Hoechst 33342	MERCK, Sigma Aldrich	
PBS	Gibco	15400-054
FITC CD105 Antibody	BioLegend	323203
AlexaFluor 700 CD73 Antibody	BioLegend	344041
Brilliant VioletTHY1 Antibody	BioLegend	328121

Methods
Ethical approval

Patient leukaemia cells were obtained from the Newcastle Biobank (REC reference number 07/H0906/109+5). All samples were acquired following written informed consent. Animal data shown here reflect unpublished data that were existing from past experiments. These experiments were carried out as per previously published methods.[12,17,25] PDX-ALL samples used in this project are from our cryopreserved PDX bank where the samples were generated in previous/earlier projects. All animal studies were performed in accordance with UK Animals (Scientific Procedures) Act, 1986 under project licence P74687DB5 following approval of Newcastle University animal ethical review body (AWERB).

2D BM-MSC-ALL co-culture

Patient derived PDX-ALL samples were cultured on 2D monolayers of MSC cells using a protocol published in an earlier study.[17] MSC used in this study are between passages ranging from p2 – p5. MSC were seeded on Matrigel coated 48 well plates at 1×10^4 cells/0.5 mL/cm^2 in their respective media (MSC media, Table 1). After 24 hours, the media was aspirated, and the cells rinsed with 1 mL SFEM. Leukaemia cells were then plated onto the MSC layers, at a density of 0.25×10^5 cells/1 mL per well, suspended in SFEM media.

3D culture of BM-MSC

MSC were transferred to a 6-well low adhesion plate and cultured in 2 mL MSC media (Table 1) for 48 hours, after which, MSC spheroids were seen to form. Spheroids obtained using this method were irregular in shape and therefore experiments were repeated and optimised using Aggrewell400 plates. Aggrewell plates were prepared by rinsing each well with 0.5 mL of Aggrewell rinsing solution, spinning at 1500 g for 5 minutes and then aspirating excess solution. MSC were seeded at varying concentrations to track effect of seeding density on spheroid size. Cells were trypsinised and seeded in single cell format on Aggrewell plates at varying seeding densities in MSC media. Aggrewell plates were spun at 100g for 3 minutes to ensure all cells reached the bottom of the microwells. After 48 hours, MSC spheroids were ready for harvest and manually transferred onto low adhesion plates. Using a stereomicroscope fitted within a Class II biological safety cabinet, the spheroids were transferred onto respective culture-ware using a Gilson p1000 pipette with the tip-end cut off using sterile scissors.

3D BM-MSC-ALL co-culture

Leukaemia cells from PDX-ALL samples were co-cultured with the MSC spheres in SFEM media, at 2×10^5 cells/mL, in 5 ml final volume, using low adhesion 48 well plates over a 5-day period. On day of harvest, the leukaemia cells and MSC spheroids were transferred into a 30 ml universal tube. This was centrifuged at 1500 RPM for 5 minutes. After aspirating the supernatant, 0.5 ml of X1 Trypsin EDTA was added and cultures incubated at 37C, 5% CO$_2$ for 2 minutes. Cells were mechanically dissociated into single cells by resuspending with a pipette. 4 ml of FBS was added and this was centrifuged at 1500 RPM for 5 minutes. Supernatant was aspirated and cells were resuspended in 1ml of fresh SFEM media. During cell count leukaemia cells were distinguished from MSC based on their morphology and size difference, the MSC are 3-4 times the size of leukaemia cells.

Dexamethasone 3D drug assay

10 million leukaemia cells were spun at 500g X 5 minutes and plated onto 3D MSC spheres in 48 well plates as described above. 5 nM dexamethasone (stock dissolved in methanol to generate 10 mM dexamethasone concentration, working concentration of dexamethasone was made by performing serial dilutions using SFEM media) was added to desired wells and leukaemia cells were cultured for 5 days. Experiments were repeated to include a total of 3 biological replicates with each replicate including 3 independent experiments.

3D Spheroid Calcein AM/Hoechst-33342 staining

MSC were seeded into Aggrewell plates to obtain a final spheroid size of 200 cells per microwell. Cells were cultured in the Aggrewell format for 3 and 7 days following which resultant spheroids were collected for the staining procedure. The calcein stain was prepared by reconstituting the lyophilized reagent to a 1mM stock in DMSO. From this, 1 μM working solution was prepared by diluting into sterile calcium free PBS. The MSC spheres were resuspended in PBS at a density of approximately 1×10^7cells/ml, to which 10 μl of 1 μM calcein stain was added to final concentration of 0.01 μM. Cells were incubated at 37°C for 20 minutes in the dark. Cells were pelleted and resuspended in pre warmed media and left for 10 minutes to ensure optimal calcein retention before imaging. Samples that were double stained with Hoechst-33342 were stained for calcein as above and following the 10 minute incubation, were stained with Hoechst-33342 at final working concentration of 10 μg/ml in pre warmed media for 20 minutes. Digital Images were captured using Leica Application Suite X (LASX) version 3.5.7.23225.

MSC Marker Antibody staining

MSC Spheroids were collected from Aggrewell plates, disrupted to single cells using a trypsin EDTA wash followed by gentle mixing. Remaining cell clumps were removed by passing sample through a 40 μm PluriStrainer. Cells were counted using a trypan blue exclusion assay. 1 million cells were resuspended in PBS to wash before blocking in 10% FBS/PBS. Cells were again spun down and resuspended in 200 μl PBS. 5 μl antibody was added and samples were incubated at room temperature for 1 hour in the dark. Following incubation, 800 μl 10% FBS/PBS was added and cells were spun down again before being resuspended in PBS for running on flow cytometer.

Extraction of mRNA

RNA was purified from cell pellets (viable cells centrifuged at 5000 g X 5 minutes followed by aspiration of supernatant media) using the RNeasy mini kit, and as per manufacturer protocols. The RNA yield and quality was checked using the nanodrop ND-1000 spectrophotometer.

cDNA synthesis

RevertAidTMH Minus First Strand cDNA Synthesis Kit was used to synthesise cDNA from the RNA isolated. 500 ng RNA was collected and added to RNase/DEPC free water to a final volume of 11 μl. 1 μl (dN)6 (200 mg/l) random hexamers was added, mixed gently by inverting the vial and briefly centrifuged. Using a GeneAmp PCR system 2700 the sample was incubated at 65°C for 5 minutes, after which the sample was immediately placed on ice, 8 μl of the master mix (Table 2) was added, the samples were vortexed and briefly centrifuged. The samples were placed back in the PCR machine to incubate at 25°C for 10 minutes, 42°C for 60 minutes and 75°C for 10 minutes to terminate the reaction. The product was either used immediately for RT-PCR or stored at -20°C for short term storage.

Table 2. cDNA master mix constituents.

Reagents	Volume (μl)
5x Reaction Buffer	4
RNase Inhibitor	1
10mM dNTP	2
RevertAid H Minus MMLV RT	1
Total Volume	**8**

Table 3. qRT-PCR mix constituents.

Reagents	Volume (µl)
SYBR Green	5
10µM Working Primer	0.3
RNase-free water	2.7
cDNA Sample	2
Total	**10**

qRT-PCR

Upon receipt, primers were reconstituted in RNase/DNase free water to a final stock concentration of 100 µM. PCR Mix (Table 3) was loaded onto PCR plate in triplicates. The plate was sealed and centrifuged for 1 minute at 1000 RPM and placed in an applied Biosystems 7900HT Sequence Detection System. ViiA7TM System was used to run the qRT-PCR plate and cycle threshold (Ct) and melting curves were obtained for analysis.

Costings analysis of in vivo versus in vitro models

Costings of *in vitro* models were estimated using list prices of media, reagents and other materials associated with developing and using the models for a 2 drug combination dose finding assay. This included 5 biological replicates, 3 experimental repeats with each having 3 technical repeats with a total number of experiments of 45. These costs were divided into the following categories: Production of clinical/patient-derived leukaemia samples; *in vitro* CRISPR/RNAi organoid generation; Dose/IC50 finding for 2 drug combination; and evaluation of drug combination.

In vivo costings were calculated using figures obtained from the Comparative Biology Centre, Newcastle University. This included fixed costs including mouse purchase from an NSG in-house colony, housing and IVIS use. Comparative *in vivo* equivalents to the *in vitro* design accounted for: production of PDX engrafted tissue (5 PDX, 6 NSG mice/PDX); *in vivo* CRISPR/RNAi (control and treatment groups, 5 NSG mice each); Dose finding for 2 drug combination (3 mice per sex); and evaluation of the combination (4 arms, 6 mice per arm). All total costings were calculated using Microsoft Excel and figures were created using Microsoft Excel.

Data analysis

Microscopy images were analysed using the Windows download bundled with Java 8. Version 1.4.3. Real time qPCR data were captured and analysed using the StepOne Plus Real-Time PCR System (ThermoFisher Catalog No: 4376600) and Software package StepOne Software v23.

Results
Development and validation of BM-MSC spheres
We find that 2D *ex vivo* human cell-based co-culture platforms potentially enable 73% (metrics obtained locally) replacement of animals in drug testing experiments. Drug testing experiments are conventionally carried out using PDX-mouse-models, currently regarded as gold standard preclinical leukaemia models (Figure 1). Here we develop 3D BM-MSC-spheroids, to improve the spatial biomimicry of functional cell-cell contacts, by subculturing MSC onto low adhesion non-tissue culture treated plates (Figure 2a-b). We show that 3D MSC retain expression of key genes such as THY1 (CD90), CDH2 and NES (Figure 2c).

Aggrewell400 plates are designed for higher throughput spheroid engineering. These plates further aid the production of MSC spheres that show greater consistency in shape and size. Each well on these plates house 400 conical microwells, funnelling single cells and encouraging them to conglomerate into spheroids (Figure 2d). Here we use these microwells to create uniform BM-MSC spheres (Figure 2e), consequently allowing standardisation of the 3D technique. We find a distinct correlation between seeding cell density and size of spheroids, with higher seeding densities resulting in generation of more uniformly sized spheroids (Figure 2f). For subsequent experiments, we use MSC spheroids of diameter 100 µM size, generated with 200 MSC seeded per microwell. In addition, we show that MSC harvested from these 3D spheroids retain the expression of MSC markers CD105, CD90 and CD73 at protein level (Figure 2g). We furthermore confirm the retention of cell viability within these spheres via Hoechst-33342 dye, which stains live and dead cells and calcein dye, which stains live cells (Figure 2h).
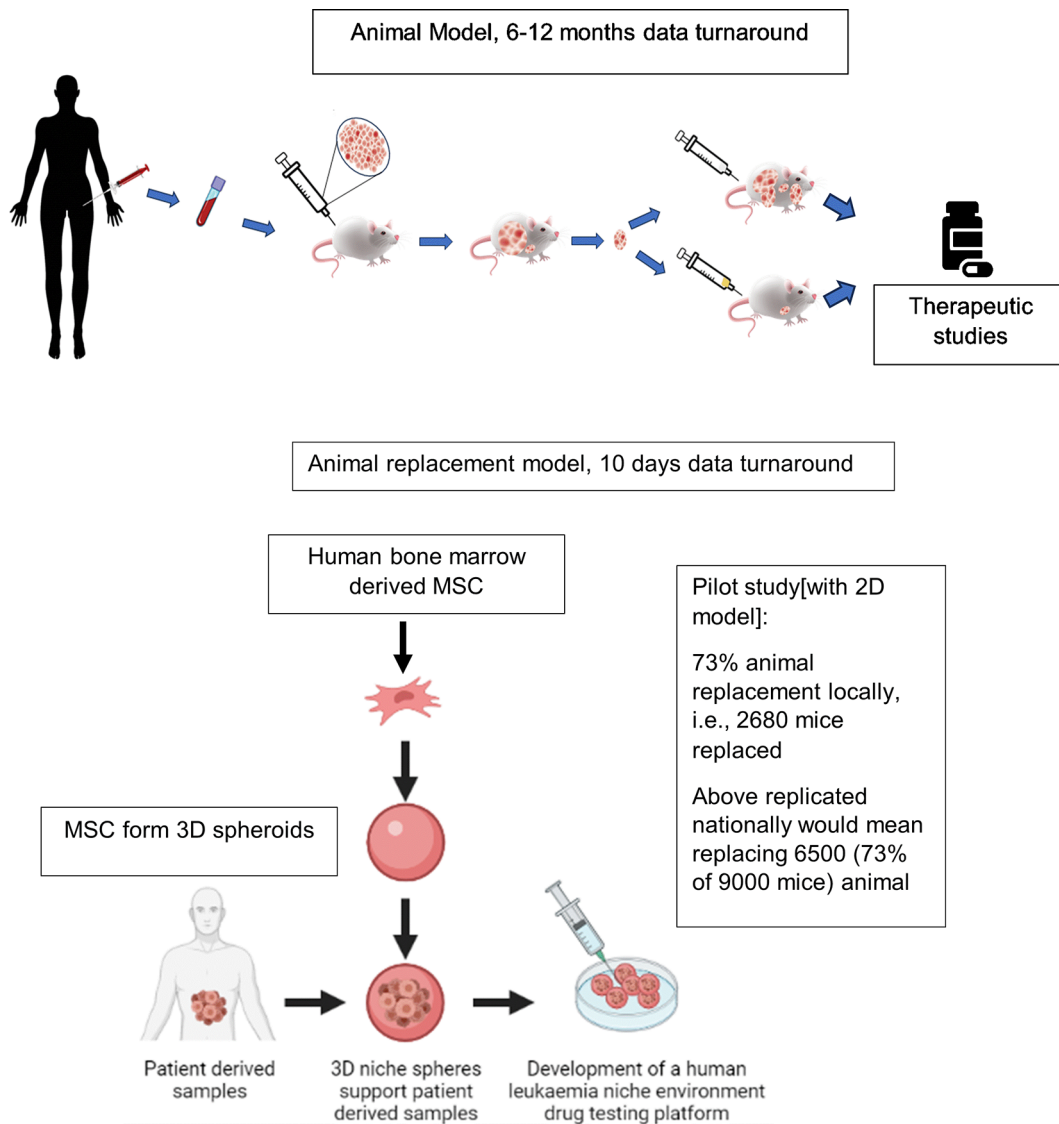
Figure 1. Comparison of existing *in vivo* preclinical leukaemia models with newly developed *ex vivo* animal replacement organoid models. Key benefits of the animal replacement organoid models include species specificity and consequently higher biomimicry.

Patient-derived ALL cells co-cultured with BM-MSC show superior proliferation and reduced dexamethasone sensitivity

We co-culture PDX-ALL cells with 2D MSC or with 3D MSC-spheroids, and monitor ALL cell counts over a 5-day period. We find that when co-cultured with MSC-spheroids, the leukaemia cells show 2-fold higher cell proliferation compared to 2D MSC-co-cultures (Figure 3a-c). Next, we repeat the co-cultures under low dose dexamethasone (5nM) treatment pressure. Dexamethasone is a glucocorticoid routinely used in the clinics to treat ALL. We find that ALL cells show greater reduction in sensitivity to dexamethasone when co-cultured with 3D BM-MSC (Figure 3d-f). This could potentially be due to improved cell-cell contacts and physiologically relevant cell polarity being achieved in a 3D format. This might have caused increased 3D versus 2D MSC-conferred ALL proliferation and/or improved ALL survival. Reduced sensitivity could also be due to altered drug availability within 3D spheroids. These data highlight the advantage of 3D organoid-based co-culture systems over routine 2D cultures in two respects: 1. Achieving higher ALL cell cycling which is essential for the action or testing of majority of anti-cancer treatments 2. Reduced drug sensitivity, possibly owing to improved PDX-ALL survival via MSC-conferred treatment protection. We further investigate the ability of MSC-spheroids to reduce ALL cell death and find that when treated with a higher dose of dexamethasone, that is, 10nM, then both 2D and 3D MSC-spheroids support survival of leukaemia cells (Figure 3g). We now progress to reveal proof-of-confidence-in-application data, justifying the suitability of our platform in conducting combinatorial drug testing.
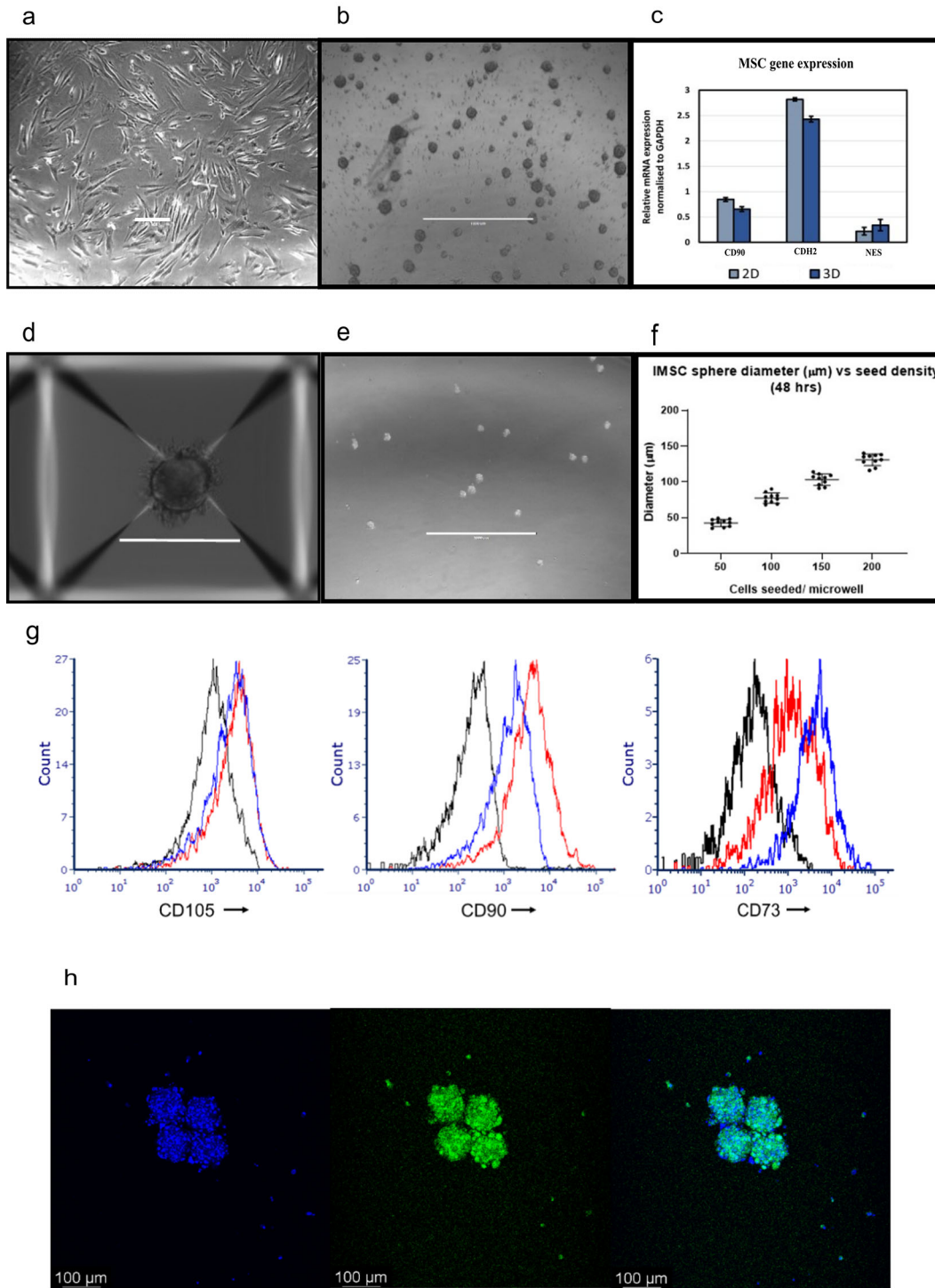
Figure 2. Aggrewell plates generate uniform 3D spheroids from single cell suspension of the desired cell-type. SD from three independent experiments (using MSC from same patient) have been plotted as error bars. Phase contrast photographs showing (a) 2D MSC, scale bar = 100 µM and (b) 3D MSC, scale bar = 200 µM after 96 hours of growth. (c) Mesenchymal marker levels quantified through qPCR and normalised to GAPDH. (d,e) Phase contrast photograph of MSC spheroids generated through culture using Aggrewell plates. Scale bar = 1000 µM (f) Correlation between cell seeding densities and size of resultant spheroids. Data captured following 48 hours of seeding. (g) CD105, CD90 and CD73 expression in MSC. Black = unstained. Blue = 2D MSC. Red = 3D MSC. (h) Hoechst-33342-Calcein staining in 100 µM spheroids, taken at day five of 3D culture. Calcein (green) stains live cells, Hoechst-33342 (blue) stains live and dead cells.
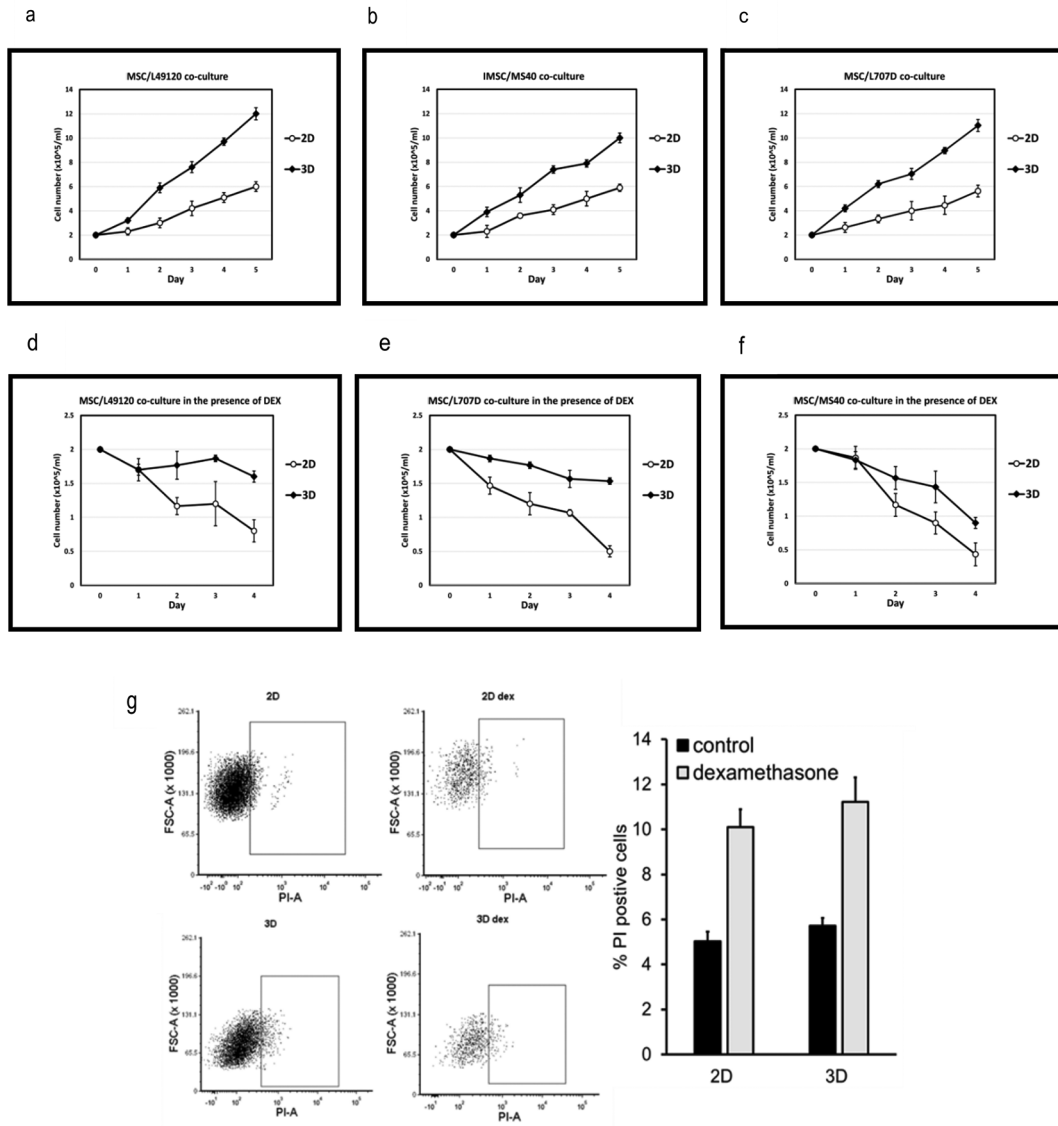
Figure 3. 3D MSC-spheroids confer higher proliferation and improved survival onto patient-derived ALL. Growth kinetics, measured via viable cell counts of ALL samples (a,d) L49120, (b,e) MS40 and (c,f) L707D across a 5 day period, with and without low dose dexamethasone (10nM) pressure, as co-cultures with 3D MSC spheres versus 2D MSC-co-culture. L49120, MS40 and L707D refer to three different patient samples, i.e., 3 biological repeats. (d,e,f) Dexamethasone treatment, data shown for 5nM treatment, is less effective in a 3D microenvironment. N= 3. (g) Flow cytometry data, representative plots, showing propidium iodide, PI, positive ALL cells (sample L707) following five days of 10nM dexamethasone treatment. The column graph shows % ALL cells stained positive for PI. N = 2.

Co-cultures of 3D BM-MSC with PDX-ALL cells how high *in vivo* predictivity

Improved *in vivo* predictivity is a key requisite for *ex vivo* animal replacement models, if these platforms are to effectively replace mouse experiments. We confirm that BM-MSC/leukaemia 2D co-culture platform generate drug dose response data that was largely comparable with drug response observed *in vivo* in PDX-mouse models (Figure 4a). Furthermore, we reveal that when PDX-ALL cells are co-cultured with 3D BM-MSC-spheroids they show greater reduction in sensitivity, compared to the 2D co-culture arm, against dexamethasone, ABT-199 (a molecularly targeted therapy against BCL-2 positive blood cancers), and dexamethasone-ABT-199 combination in a sample subgroup that exhibited reduced *in vivo* efficacy (obtained from previously unpublished *in vivo* data existing in the lab from earlier studies). This shows the ability of our 3D co-culture model to screen off false-positive hits derived from 2D models, consequently minimising *in vitro* to *in vivo* attrition rates. We further confirm that when co-cultured with 3D MSC spheres, ALL cells expectedly
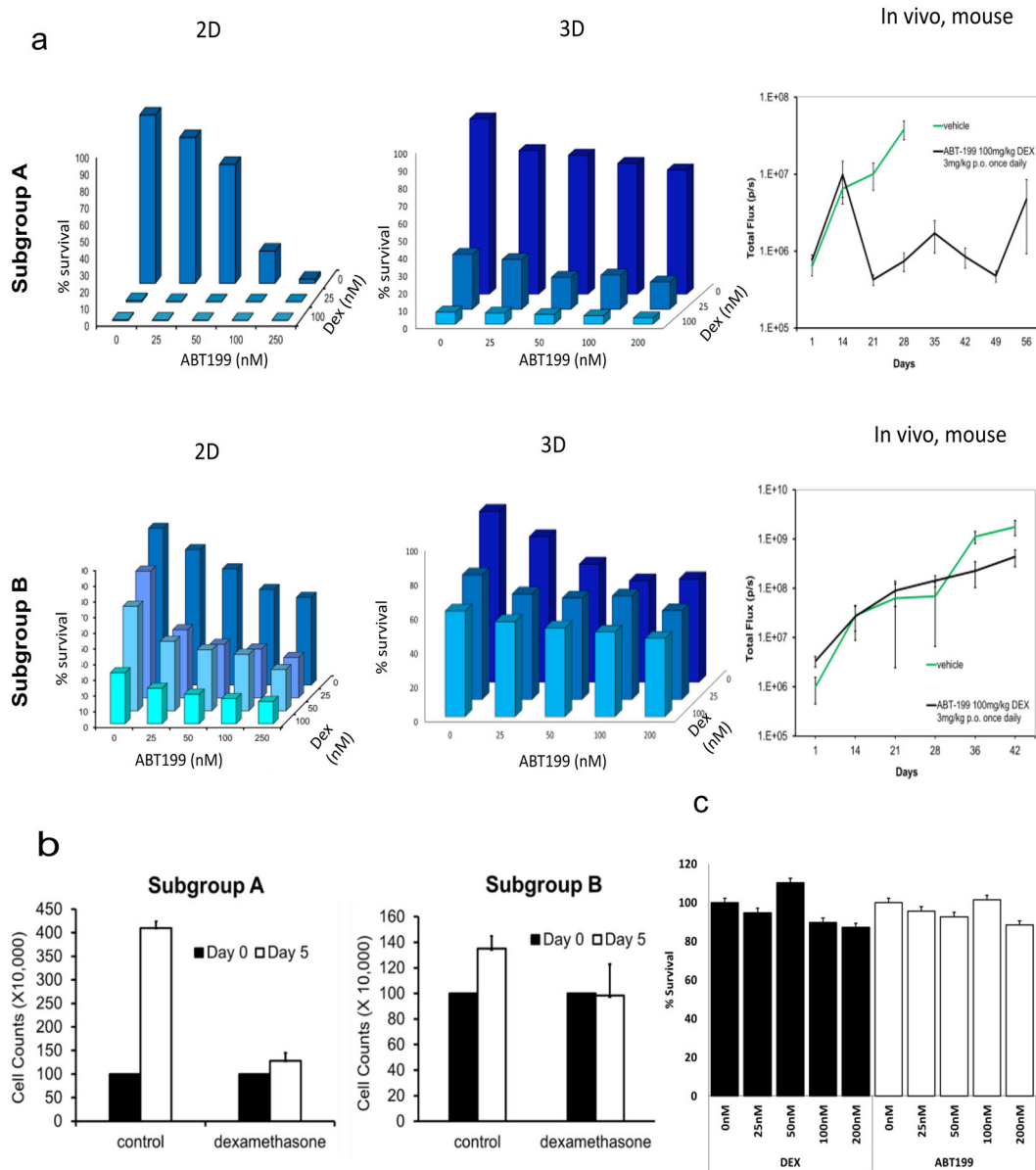
Figure 4. *In vitro* co-culture platform delivers drug response data with high *in vivo* predictivity to minimise *in vitro* to *in vivo* drug attrition rates. Leukaemia cells from leukaemia cytogenetic subgroup A is a responder to ABT199 (targets BCL2 in BCL2 positive ALL) and dexamethasone drug combination *in vitro* and *in vivo*. On the other hand, leukaemia cells from subgroup B show low sensitivity to this drug combination *in vitro* in both 2D and 3D formats, and subsequently poor response is also noted *in vivo* using PDX mice models. *In vitro* data readout includes viable cell counts; *in vivo* data (right hand graphs) is shown as bioluminescence imaging from luciferase expressing PDX cells in total flux(p/s). Total flux is a surrogate of blast number *in vivo*. Error bars shown refer to standard error (SE) using 4 mice per treatment group. Dexamethasone and ABT-199 are used in the clinics to treat ALL. b. ALL cell counts on day 0 versus day 5 of culture, with and without treatment with 25 nM dexamethasone. N = 3. Error bars = SD. c. Cell counts following dexamethasone and ABT-199 treatment of 3D MSC.

proliferate in 3D-MSC spheroid-co-cultures, however, fail to increase in cell counts on day five, in the dexamethasone treatment arm (Figure 4b). Moreover, we show that neither dexamethasone, nor ABT-199, have any effect on survival of MSC, when treated for five days within a 3D sphere format (Figure 4c). Finally, we perform a costs analysis and show improved financial sustainability, namely a 30% costs benefit, in our *ex vivo* organoid platform compared to animal models (Figure 5).
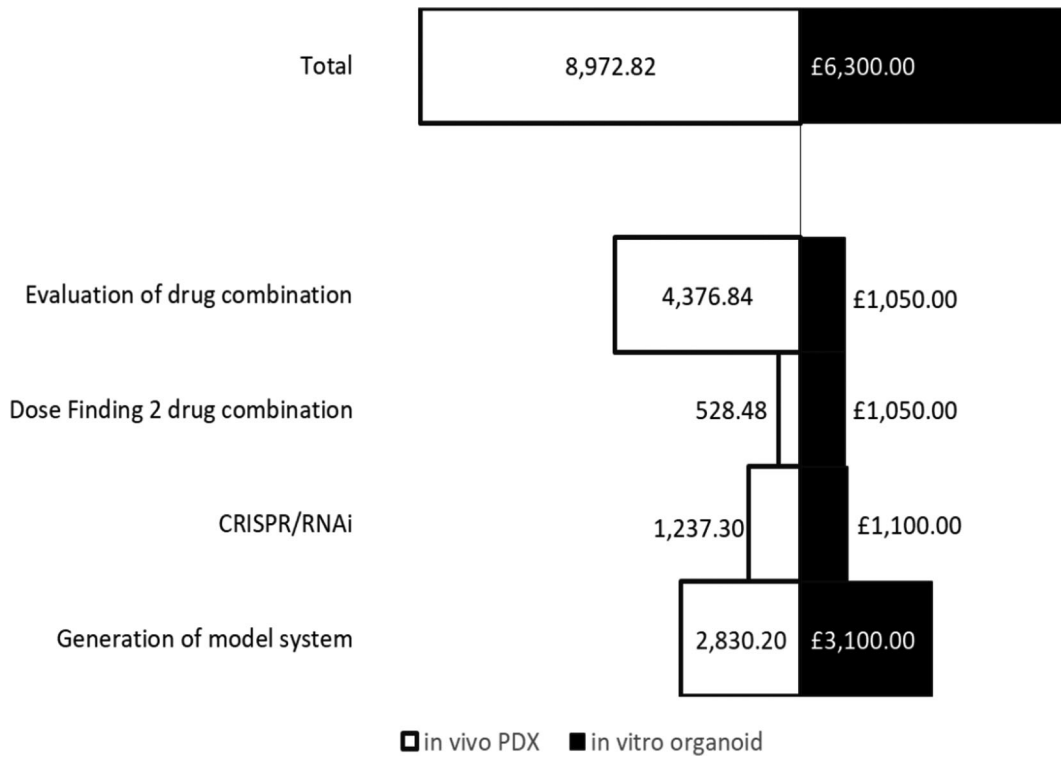
Figure 5. A comparative costs analysis between 3D *ex vivo* and *in vivo* preclinical models show an approximate 30% costs benefit and consequently increased financial sustainability when using *ex vivo* organoid models.

Discussion

Patient stratification and precision oncology are emerging as mainstay to treat disease in the most effective way possible, allowing weaknesses in tumour biology to be exploited therapeutically. However, high drug attrition rates remain a challenge when developing new treatments in ALL. High drug attrition has been attributed to preclinical models not being clinically translatable. Key barriers hindering bench to bedside translation when using mouse models include species specificity, high financial costs, and lengthy experiments. Not all leukaemia samples engraft in mice. We have shown, in a previous study, that the 2D MSC co-culture system can successfully culture acute myeloid leukaemia (AML) cells from t(8;21)-positive samples,[7] which failed to engraft any mouse model available at the time. Moreover, samples that do engraft form successful xenograft models, yielding PDX samples in 3-6 months at which point drug testing can be started, which subsequently takes another 3-4 weeks. Indeed, although the time taken to generate a novel PDX-ALL sample can span between months to a year, this time is often shortened when cells from established PDX-ALL samples are serially transplanted in subsequent mouse models, to aid the generation of PDX-ALL lines. Nevertheless, some samples, particularly those belonging to good risk groups, such as high hyperdiploid samples, still require between 6 months – 1 year to engraft successfully before any drug response testing can begin. Moreover although *in vivo* toxicity assays are not needed for drugs being re-purposed, indicating this to be an area where animal-replacement technologies may perhaps not be relevant, animal testing has been required prior to regulatory approval being given for first-in-human studies. Furthermore, toxicity studies which use animal models are indicated when testing new combinatorial treatment regimens comprising repurposed drugs. This is because tolerability of drug combinations can be distinct from that of component single drugs.

Nevertheless, the U.S. Food and Drug Administration (FDA) has recently announced that new drugs would not be mandated to be tested in animals prior to first-in-human trials, and an FDA-wide program is furthermore prioritising development of human-relevant methods, with high *in vivo* predictivity, to replace, reduce and refine animal models in drug testing. This further corroborates the timeliness of prioritising development of transformative human cell-based, non-animal technologies towards preclinical drug testing. Most specifically, a key advantage of our prototype approach would be in medium-high throughput screening of single and combinatorial compounds, particularly as pre-*in vivo* screens, to prevent "false positive" 2D *in vitro* hits, such as that observed in Figure 4, from progressing towards *in vivo* drug efficacy testing.

A 3D BM-mesenchyme-leukaemia spheroid model will provide researchers with a tractable platform with improved biomimicry, where clinical samples can be cultured within the context of their mesenchymal microenvironment, and patient specific drug testing can be performed within shorter time periods, such as a week. In circumstances where sourcing primary MSC may be a challenge, human induced pluripotent stem cell (iPSC) differentiation into MSC may be an attractive alternative.[12] Besides delivering drug response data within clinically relevant timeframes the relative simplicity of human-cell-based spheroid models mean that they are tractable, transferrable, and sustainable. Consequently, such models have wide applications in translational research in academia and industry alike and can be set up in laboratories and SMEs locally, nationally and globally, especially in organisations that do not contain infrastructure for animal procedures. Following further extensive validation, such 3D-based preclinical models would furthermore have the potential to be embedded within clinical trials and healthcare systems for the purposes of detecting responders as well as to aid risk stratification. In this paper we show that our 2D model itself brought about significant animal replacement locally. Leukaemia research at Newcastle requires approximately 3600 animal procedures every year. Local metrics from previous studies confirmed that using the 2D pilot approach we replaced mice in approximately 67 drug tests each year. Given minimum of 40 animals are needed per drug test we replaced 2680 animals (out of 3600) which constituted a minimum 73% local animal replacement. To improve biomimicry and consequently translatability and transferability of our model, here we develop 3D BM-mesenchyme spheroids. We show that these 3D spheroids show superior ability in supporting culture of patient-derived ALL samples. We also show that sensitivity of leukaemia cells to drugs such as dexamethasone is reduced when these cells are being co-cultured with 3D spheroids. This means that compared to 2D MSC cultures, MSC spheroid-based models, might be promising to study the biology of leukaemia cells from relapsed disease, consequently helping interrogate treatment resistance mechanisms. Treatment resistance remains a major clinical challenge in cancer management. This simple and tractable 3D prototype will form the first steppingstone in developing next generation, scalable 3D preclinical models with improved *in vivo* and patient drug response predictivity. Such sustainable *ex vivo* models will ultimately aim to successfully replace existing moderate severity animal procedures in leukaemia research with clinically relevant human cell-based models that are able to successfully impact positive clinical outcome.

Study limitations

Here we use primary human MSC, which indeed may be difficult to source, and which may lead to donor variability. Using iPSC derived MSC to co-culture patient-derived leukaemia[12] may be a more suitable alternative, and would furthermore enable assay standardisation, thereby improving data reproducibility. Moreover, although we show that MSC showed no adverse effect following dexamethasone and ABT-199 treatment, many anti-cancer treatments notably cytotoxic chemotherapy may cause direct toxicity to the MSC. However, this further emphasises the need to include key microenvironment cell types when conducting anti-cancer drug testing, especially given cancer treatments influence interactions between leukaemia and their surrounding niche, with many cytotoxic treatments affecting both leukaemia and BM cells in patients. Furthermore, we acknowledge that an ideal representation of 3D biomimetic human cell-based model would include capturing both mesenchymal and vascular BM components within an immune-responsive context.

Data availability

Mendeley Data. A human mesenchymal spheroid prototype to replace moderate severity animal procedures in leukaemia drug testing. https://doi.org/10.17632/56npmkbpfb.1.[24]

This project contains the following underlying data:

- - 3D spheres.tif

- - 3D Sphere.tif

- - drug combination.xlsx

- - *in vivo* Vs *in vitro* costs.xlsx

- - Raw Figure Data.xlsx

Acknowledgements

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Version 2

Reviewer Report 20 November 2023

https://doi.org/10.5256/f1000research.157299.r221081

✔ **Simon E. Richardson** (iD)

Wellcome - MRC Cambridge Stem Cell Institute, Cambridge, UK

Many thanks for accommodating my recommendations.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Leukaemia biology, disease modelling, stem cells, epigenetics, preclinical drug testing.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 31 January 2023

https://doi.org/10.5256/f1000research.135152.r159730

❓ **Helen Wheadon**

Paul O'Gorman Leukaemia Research Centre, School of Cancer Sciences, University of Glasgow, Glasgow, UK

The manuscript by Wilson *et al.* provides a convincing argument for the requirement of additional in vitro models to improve drug testing in acute lymphocytic leukaemia (ALL) and reduce the

reliance on Xenograft mouse models. Preliminary data is presented supporting the use of 3D human mesenchymal stroma cell (MSC) spheroid/primary ALL co-cultures for the use in preclinical testing of drugs. Proof of principle data is provided showing that simple 2D co-culture on MSCs gives a similar range of drug responses to dexamethasone and the BCL2 mimetic drug ABT199 as observed in in vivo studies for 2 primary ALL patient samples. The authors then show optimisation data using Aggrewell plates, demonstrating uniform spheroid formation and a corresponding increase in spheroid diameter when cell seeding density is increased. The final figure then compares primary ALL cell proliferation in 2D and 3D culture and drug responses to dexamethasone. Using 3 independent patient samples they show that MSC spheroids support cell proliferation over a 5 day time frame and provide protection to dexamethasone. These are important observations and support the introduction of a more physiologically relevant humanised system for drug screening prior to conducting in vivo studies. Another potential advantage would be if the authors had data demonstrating that the system supports patients' samples which do not engraft, as there is a high engraftment failure rate in Xenograft models with not all patient samples being successful. This could potentially lead to more robust data on patient's responses. Given patient heterogeneity mouse models may be biasing certain traits/genetics due to the engraftment limitations observed.

Overall, the system if adopted by other researchers could potentially reduce animal numbers and suffering, reduce the cost and select the most promising drugs for additional testing. It would therefore have impact on replacement and reduction of mouse experiments.

However, there are some limitations in the results shown that would be strengthened by additional experiments:
1. The MSC are characterised by gene expression, it would be good to include flow cytometry data; $CD90^+105^+73^+34^-45^-$ are key markers demonstrating stemness. The culture conditions using FCS can also lead to changes in the MSCs so it would be good to include passage number. Figure 3 live/dead staining of the spheroids especially for the size used in the drug treatment assays in Figure 4 would be important to include.

2. Data showing that dexamethasone and ABT199 do not detrimentally affect the MSC monoculture and MSC spheroids would also be important to include.

3. Figure 4 looks at proliferation, apoptosis assays would be good to include to show whether cell death was occurring.

4. Data showing similar results in ALL patient samples known to fail to engraft in vivo would strengthen the rationale for adopting the 3D system.

5. Although the potential costs and animal savings presented are feasible, the system is quite simplistic and is more likely to be incorporated as an additional test rather than be adopted as a replacement. It would be more attractive if the time frame could be extended and more details on the application for high throughput screening development was discussed. Also, in vivo toxicity testing of novel compounds will still be required in order for regulatory approval for any Phase 1 clinical trial in humans to be progressed.

Minor corrections:

Research highlights - blasts would only be appropriate for certain types of leukaemia, either

need to define it's ALL specifically or change to stem/progenitor.

Scientific benefit:
A 3D spheroid-based approach with improved clinical relevance for ex vivo co-culture of primary patient leukaemia samples for further investigation into cancer biology such as stem/progenitor cell-niche interactions, stem/progenitor cell proliferation and treatment resistance.

3Rs benefit:
To replace moderate severity animal (mice) procedures in leukaemia research and early pre-clinical drug testing.

Current applications:
Exploration of leukaemia biology such as stem/progenitor cell proliferation, stem/progenitor cell - niche interactions, niche-impacted treatment resistance and obtain drug response data.

Introduction:
- ○ Need to define at the start that you are talking about ALL as it reads like a general overview which means some of the % incidence rates etc. are not correct. It would be good to mention what type of ALL as well, is it B-ALL, T-ALL, childhood, adult?

- ○ Paragraph 3 page 3: Most research groups would use primary patient samples in vitro prior to moving to in vivo models. This should be included.

- ○ Paragraph 2 Page 4: Remove blasts and replace with LSCs also include disease type chronic myeloid leukaemia.

- ○ Page 11 'such spheroid biobanks': do they actually exist? Or are you planning on constructing a biobank?

- ○ 'MSC spheroids provide superior protection to leukaemia cells against dexamethasone treatment. are appropriate in modelling treatment resistance which remains a major clinical challenge in cancer management'.
  Is this statement correct, not really looking at resistance, just reduced sensitivity to the drugs at that dose when in 3D rather than 2D culture. Need to amend and extend to include how you would look at resistance i.e. relapsed patients and the potential to interrogate resistance mechanisms.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Stem cells, leukaemia modelling, signalling, pre-clinical drug testing.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 19 December 2022

https://doi.org/10.5256/f1000research.135152.r158193

? **Simon E. Richardson** iD
Wellcome - MRC Cambridge Stem Cell Institute, Cambridge, UK

This manuscript by Wilson et al provides a rationale, methodological details and pilot data supporting the use of 3D human mesenchymal stroma cell (MSC) spheroid co-cultures in the preclinical testing of drugs for acute leukaemia. The authors provide a detailed rationale for the potential benefits of this system, including: i) reduction in animal use/suffering; ii) reduced costs; and iii) the ability to model the protective impact of bone marrow micro-environmental influences in abrogating drug efficacy in vitro, reducing the number "false positive" drug hits taken forward to in vivo model systems. The authors provide data showing the equivalence of 2D in vitro drug sensitivity with PDX models (n=2) and compare 2D vs 3D co-culture of human leukaemias (n=3) in proliferation and dexamethasone sensitivity assays, showing consistently enhanced proliferation and reduced dexamethasone sensitivity in the 3D system. They also present methodological data on the establishment of MSC spheroids as well as modelling on the potential reduction in animal use and costs based on local experience.

Overall, the rationale to develop more physiologically relevant in vitro co-culture systems is a very worthwhile endeavour with clear potential benefits for animal welfare, costs and in streamlining drug development. It also has the potential advantage of being used in the study of leukaemias that do not engraft in current immunosuppressed mouse models.

In my opinion the work has three major limitations that would benefit from either additional data or acknowledgement in the discussion.

1. Whilst this work demonstrates the feasibility of the approach, it is not possible from the data presented to conclude that 3D systems perform better than either 2D co-culture or PDX systems. Specifically, the data presented uses a small number of patient samples (n=2 or 3) and drugs (Dexamethasone ± ABT199). The three systems are not directly compared and no example of an in vitro drug hit that proves "false-positive" when tested in vivo is tested in parallel 2D/3D co-culture.

2. There are a number of potential technical limitations of the co-culture approach that are not acknowledged: i) the requirement for access to a source of MSCs which are not readily available to most labs; ii) certain drugs (notably cytotoxic chemotherapy) are likely to have direct toxicity to the MSCs themselves; iii) the lack of a vascular component to this system might result in artificially high levels of physical protection from drug exposure by the 3D niche (i.e. potential for false negatives).

3. The potential costs and animal savings presented are feasible, but likely at the upper end of the spectrum and will differ between institutions. For example, the time to generate a novel PDX is indeed many months, but once established PDX models in ALL engraft much more quickly and reproducibly and established PDX material is readily available. In vivo toxicity testing is not needed for established drugs being re-purposed; conversely novel chemical agents will still need some in vivo toxicity data prior to regulatory approval for first-in-human studies.

Minor points:
1. In the introduction it would be helpful to specify the precise leukaemia being studied and at which age. In particular, relapse rates in adult B-ALL are much higher than those presented, which are indicative of the paediatric setting.

2. In the 2D co-culture methods, please provide a reference on how to process MSCs and specify the final volume of media per well used to plate the leukaemia cells.

3. For the response data presented in figures 1 and 4, please clarify in the legend or methods the nature of the readouts and timepoints used.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Leukaemia biology, disease modelling, stem cells, epigenetics, preclinical drug testing.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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