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Leukemic blasts program bone marrow adipocytes to generate a pro-tumoral microenvironment

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Runinng title: Bone marrow adipocytes drive leukemic cell proliferation

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Key Points

- Bone marrow adipocytes support AML survival.
- AML induces adipocyte lipolysis of triglyceride to free fatty acids and subsequent transport by FABP4.

Abstract

Despite currently available therapies most patients diagnosed with acute myeloid leukemia (AML) die of their disease. Tumor-host interactions are critical for the survival and proliferation of cancer cells; accordingly, we hypothesise that specific targeting of the tumor microenvironment may constitute an alternative or additional strategy to conventional tumor-directed chemotherapy. Since adipocytes have been shown to promote breast and prostate cancer proliferation, and because the bone marrow adipose tissue (MAT) accounts for up to 70% of bone marrow volume in adult humans, we examined the adipocyte-leukaemia cell interactions to determine if they are essential for the growth and survival of AML. Using in-vivo and in-vitro models of AML we show that bone marrow adipocytes from the tumor microenvironment support the survival and proliferation of malignant cells from patients with AML. We show that AML blasts alter metabolic processes in adipocytes to induce phosphorylation of hormone-sensitive lipase and consequently activate lipolysis, which then enables the transfer of fatty acids from adipocytes to AML blasts. In addition, we report that fatty acid binding protein-4 (FABP4) mRNA is up-regulated in adipocytes and AML when in co-culture. FABP4 inhibition using FABP4 shRNA knockdown or a small molecule inhibitor prevents AML proliferation on adipocytes. Moreover, knockdown of FABP4 increases survival in Hoxa9/Meis1-driven AML model. Finally, knockdown of carnitine palmitoyltransferase IA (CPT1A) in an AML patient-derived xenograft model improves survival. Here we report the first description of AML programming bone marrow adipocytes to generate a pro-tumoral microenvironment.

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Introduction

Survival of patients with acute myeloid leukemia (AML) is poor; two-thirds of young adults and 90% of older adults die of their disease.[1] Improved outcomes will now only come from novel treatment strategies derived from an improved understanding of the biology of the disease.

All mammals generate blood within their bones and AML arises from myeloid progenitors within the bone marrow (BM) microenvironment. AML blasts exhibit high levels of spontaneous apoptosis when cultured in vitro but have a prolonged survival time in vivo further indicating that the tissue microenvironment plays a critical role in promoting and maintaining AML cell survival.[2-5] In human leukemias the microenvironment provides a number of soluble factors whose primary functions are to promote survival and homing,[6,7] which implies that the apoptotic defect in AML is not cell-autonomous but highly dependent on extrinsic signals derived from the cellular microenvironment. These complex cell-cell interactions between AML blasts and the cells that support them within the bone marrow may therefore provide an attractive target for novel drug therapies.

The BM microenvironment is defined by cell types not directly involved in haematopoiesis and include: macrophages; endothelial cells; osteoclasts; osteoblasts; adipocytes and fibroblasts. BM adipocytes were identified over a century ago, and bone marrow adipose tissue (MAT) accounts for up to 70% of BM volume of the axial skeleton in adult humans.[8-10] MAT is an energy storage organ and consists primarily of triglycerides which can be broken down to release free fatty acids (FA) which in turn can be used to generate ATP. In cancer, a recent study by Cawthorn et al (2014), demonstrated that BM adiposity and circulating adiponectin increase in cancer therapy.[8] Moreover, TNF and adiponectin from the BM of patients with AML inhibit normal hematopoiesis.[11] However, although AML blasts are highly proliferative and grow within an adipocyte-rich environment, to date there is limited knowledge regarding the specific functions of BM adipocytes in relation to the proliferation and survival of AML.

Adipocytes, in the context of solid malignancies, support tumor survival, proliferation and metastasis.[12-14] In a study on breast cancer and the associated adipocytes

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therein, Dirat et al. (2011) showed that these cancer associated adipocytes are greatly influenced by the invasive cancer cells with which they are associated.[12] This study supports the concept of cancer cells orchestrating an environment which favours their progression through complex mechanisms by obtaining adipocyte participation. Furthermore, studies carried out in prostate cancer have demonstrated lipid transport between adipocytes and prostate cancer cells.[13,15] In models of bone metastasis following prostate cancer, Herroon et al. (2013), show functional evidence of BM adipocyte support of tumor growth and identify fatty acid binding protein-4 (FABP4) as a key protein involved in the mechanism. While this study investigates FABP4 expression in the cancer cells, a separate study has investigated FABP4 expression in ovarian cancer associated adipocytes.[14] FABP4 upregulation has been shown in the fat-rich omental metastases compared to the primary tumor site and mice lacking FABP4 show significant reduction in metastatic tumor growth, indicating the role of FABP4 in cancer proliferation and metastasis. Taken together, these studies identify a functional role for adipocytes and fatty acid transporters in the support of solid tumor metabolism and subsequent metastatic spread.

In the present study, we look to identify whether adipocytes directly support the proliferation of human AML. Furthermore we evaluate the mechanisms controlling the interaction between AML blasts and MAT, as well as the downstream metabolic consequences in both the adipocytes and leukemia cells. Finally, we identify key regulators in the AML/MAT interaction and assess AML survival following targeted inhibition of this interaction.

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Materials and Methods

Materials

Anti-phosphorylated HSL, anti-HSL, anti-HIS and anti-FABP4 were purchased from Cell Signalling Technology (Cambridge, MA, USA, Cat. 4126, 4107, 2365 and 3544 respectively). Control-IgG-FITC, control-IgG-PE, control-IgG-APC, anti-CD34-PE, anti-CD90-FITC, anti-CD73-PE, anti-CD105-APC, anti-CD33-APC and anti-CD45-FITC, antibodies were from Miltenyi Biotec (Bergisch Gladbach, Germany, Cat. 130098847, 130098845, 130092214, 130098139, 130095403, 130095182, 130094926, 130098043, 130098864). Lineage Cell Depletion Kit (mouse) was also purchased from Miltenyi Biotech (Cat 130090858). Acipomox, etomoxir and FABP4 inhibitor were purchased from R&D Systems (Abingdon, UK, Cat. 2784 and 4539). Recombinant FABP4-his was purchased from Abcam (Cambridge, UK Cat. 204760). All other reagents were obtained from Sigma-Aldrich (St Louis, MO, USA), unless otherwise indicated.

Primary cell culture and differentiation

Primary AML blasts and non-malignant CD34+ cells were obtained from patient bone marrow or blood following informed consent and under approval from the UK National Research Ethics Service (LRECref07/H0310/146). Cell isolation was carried out by density gradient centrifugation using Histopaque (Sigma-Aldrich, Cat. 1077) and cell type was confirmed by flow cytometry. All cells were grown in normal media (Dulbecco's Modified Eagle's Medium (DMEM) (ThermoFisher, Cat. 21885-025) supplemented with 20% foetal bovine serum (FBS) (ThermoFisher, Cat.10082139)) with or without the addition of cytokines purchased from Peprotech, UK, 100 ng/ml SCF (300-07), 50 ng/ml FLT3L (300-19), 20 ng/ml IL-3 (200-03) and 20 ng/ml G-CSF (300-23). Bone marrow stromal cells (BMSC) were isolated from AML bone marrow samples by adherence to tissue culture plastic and were then expanded in DMEM containing 20% FBS and supplemented with 1% penicillin-streptomycin (ThermoFisher, Cat 15140122). BMSC markers were confirmed using flow cytometry for expression of CD90+, CD73+, CD105+ and CD45-. [16] To induce adipocyte differentiation of BMSC (passage 2-4), a cocktail of dexamethasone (1μM), indomethacin (0.2mM), insulin (100nM) and 3-isobutyl-1-methylxanthine (0.5mM) (Cat. D4902, 17378, I9278 and I7018) in DMEM containing 10% FBS was prepared. The differentiation of BMSC to adipocytes was between 75%-95% as measured by

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neutral lipid specific BODIPY® 493/503 dye (supplementary figure 1A and supplementary table 1). After differentiation, adipocytes and control BMSC were analysed for markers of adipocytes (adiponectin, FABP4 and CEBPa). Figure 3A and Supplementary figure 1B shows that differentiated adipocytes express higher levels of adiponectin, FABP4 and CEBPa than BMSC.

Flow cytometry

For this study we used the Accuri C6 (Bectin Deckinson, Oxford, UK) and the CyFlow Cube 6 (Sysmex, Milton Keynes, UK) for flow cytometry analysis. Cells were incubated for 5 minutes with the FCR receptor blocker (Miltenyi Biotec, Cat. 130-059-901) and then stained with isotype controls or test antibodies (Miltenyi Biotec). Gates were set to the appropriate isotype control.

Co-culture assay

BMSC were seeded at 3 $x10^4$ cells per well of a 12 well plate and $1x10^4$ for a 24 well plate in normal growth media. Cells were left to adhere and proliferate until maximum confluency. Growth media was replaced with differentiation media which was replaced every four days until day 21. Differentiated adipocytes were washed twice with normal growth media and then placed in normal growth media until co-culture experiment. For co-culture experiments AML cells were placed on the adipocytes at $5x10^5$ (per well of 12 well plate) and $1x10^5$ (per well of a 24 well plate) in normal growth media. Adipocyte/AML co-cultures were then incubated for indicated time points and Supplementary Table 2 shows the combination of different samples used for each experiment. All co-cultures were from different patients, at no point were autologous co-cultures performed. To isolate adipocytes from co-culture experiments, all suspension cells were removed by gentle pipetting and washing with PBS. This was then followed by light trypsinisation where diluted typsin with PBS (1:1) for 40 seconds followed by gentle tapping. All suspension cells where removed and adhered adipocytes were washed with PBS and then lysed using RNA or protein lysis buffer.

Lentiviral knockdown

MISSION pLKO.1-puro Control Vector, catalogue number SHC001 was used as the lentivirus control (Control shRNA). Plasmids containing MISSION® shRNA

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TRCN0000418254 (human FABP4 shRNA), TRCN0000105185 (mouse FABP4 shRNA), TRCN0000036279 (human CPT1A shRNA), TRCN and were purchased from Sigma-Aldrich and viruses were produced as previously described.[17] Lentiviral stocks were concentrated using Amicon® Ultra centrifugal filters (Cat. UFC910024) and titres were determined using Lenti-X™ qRT-PCR titration kit (CloneTech Cat. 631235). Stromal cells were plated at a density of $3x10^4$/well in a 12 well plate, expanded and differentiated into adipocytes. Adipocytes were then infected with FABP4 shRNA lentiviral stock at an MOI of 20. Human and mouse AML cells were infected with respective lentiviral stocks at an MOI of 20 in the presence of polybrene (10µg/ml final). Infected cells were analysed using real-time PCR (Roche) and Western blotting.

Patient-derived AML xenograft

For this study the NOD.Cg-Prkdcscid IL2rgtm1Wjl/SzJ (NSG) from The Jackson Laboratory, Bar Harbour, ME, USA were used. The NSG mice were maintained under specific pathogen-free conditions in the research animal facility of The Disease Modelling Unit, The University of East Anglia, Norwich, UK. All animal experiments were performed in accordance with UK Home Office regulations.

Engraftment of primary AML cells in NSG mice

$6\text{-}8x10^6$ isolated AML blasts were grown in mono culture and $2x10^6$ isolated AML blasts grown on adipocytes for 6 days, $2x10^6$ viable AML cells were washed and subsequently resuspended in PBS. Cells were then injected into the tail vein of non-irradiated 6-8 week old female NSG mice. When clinical signs of illness became apparent (rough fur, hunchback, or reduced motility) or if 12 weeks post injection was reached, mice were sacrificed by exposure to CO_2. BM and spleen were harvested and analysed for human CD33 and CD45. If more than 1% of human CD45/CD33 cells were detected in the BM or spleen, the AML sample was said to be engrafted. For AML grown in cytokine supplemented media, $2x10^6$ viable AML grown in normal media with the addition of cytokines (100 ng/ml SCF (Peprotech 300-07), 50 ng/ml FLT3L (Peprotech 300-19), 20 ng/ml IL-3 (Peprotech 200-03) and 20 ng/ml G-CSF (Peprotech 300-23).

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For the CPT1-KD experiment, isolated primary AML were grown on adipocytes for 6 days. The AML cells were removed from the adipoyctes and then infected with a control-shRNA or CPT1-shRNA lentivirus and after 96 hours cells were expanded on the adipocytes and $2x10^6$ primary AML cells were then injected into the tail vein of non-irradiated 6-8 week old female NSG mice.

Retroviral AML transplantation mouse model

C57BL/6J mice were obtained from Janvier-Labs (Le Genest-Saint-Isle, France). All animal experiments were performed according to national and international standards and had been approved. Bone marrow cells were harvested from mice, and lineage-negative cells were obtained by negative selection using the Lineage Cell Depletion Kit (mouse) as recommended by the manufacturer. Lineage-negative cells derived from C57BL/6J were retrovirally infected by co-culture with GP+E86 cells in the presence of polybrene (10 µg/ml, Sigma-Aldrich, Munich, Germany). Co-culture with GP+E86 packaging MSCV-Hoxa9-PGK-neo was performed for 3 days followed by co-incubation with GP+E86 MSCV-Meis1-IRES-YFP for 1 day. Hoxa9 cells were selected with 0.6 mg/ml G418 (Sigma-Aldrich) for at least 5 days. After selection, cells were sorted with a FACS BD Aria III cell sorter. Lentiviral transductions of cultured cells with vectors encoding FABP4-specific shRNA and control shRNA were performed as described previously.[18] $8x10^4$ cells were transplanted together with $2x10^5$ support cells by injection into the tail vein of lethally irradiated (9.5 Gy) recipient mice (C57BL/6J). Support cells were isolated from C57BL/6J mice and purified on a Ficoll gradient. To perform in vitro assays with Hoxa9/Meis1 expressing cells, cells were cultured in DMEM supplemented with 20% FBS and supplemented with the following cytokines murine SCF (cat. 250-03; 100ng/ml final), IL-6 (cat. 200-06; 10ng/ml final) and IL-3 (cat.213-13; 10ng/ml final).

Immunocytochemistry

AML-adipocyte niches formed in the BM were isolated from patient samples and grown on chambered tissue culture treated slides (12 well chamber, removable – Ibidi cat. 81201). Niches were then fixed with 4% PFA, permeabilised with 0.01% Triton and blocked with goat serum. Cells were stained with neutral lipid specific BODIPY® 493/503 (4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene) dye, CD90 and DAPI. AML blasts were removed and stained with neutral

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lipid specific BODIPY® 493/503 and CD34 antibody. Images were visualised with secondary Alexa Flour 568 or 488 conjugate immunoglobulin G (Invitrogen). Nuclei were stained with 4'6-diamidino-2-phenylindole before samples were mounted with Fluoromount aqueous mounting medium (Sigma Cat. F4680). Cells were imaged by an AxioCam ICm1 monochrome CCD camera attached to the Apotome.2 Imaging System and confocal microscopy (Zeiss LSM 800 with Airyscan) using Axiovision 4.8.2 software (Carl Zeiss). Image staining intensities were analysed with ImageJ software.

Proliferation, cell cycle and death assays

Treated primary AML blasts were incubated with 5-bromo-2'–deoxyuridine (BrdU staining kit, eBioscience, Cat. 8811-6600) and CD34 antibody. The proliferation percentage of cycling AML blasts were analysed using flow cytometry. Cell cycle analysis of AMLs from monoculture and co-culture was carried out by ethanol fixation and propidium iodide staining and quantified using flow cytometry. AML apoptosis was measured using PI/AnnexinV (eBiosciences, Cat. 88-8005-72) and was also quantified using flow cytometry.

Free fatty acid and glycerol detection

Free fatty acid and glycerol detection was performed using a lipolysis assay kit for detection of both free glycerol and fatty acids (Zenbio, Research Triangle, NC, Cat. LIP-2-NC). AMLs were cultured with adipocytes in LIP2/3 assay buffer and incubated for 24 hours at 37C. Media was assayed to detect free fatty acid and glycerol according to manufacturer's specifications.

Free fatty acid uptake assay

For lipid visualisation and transfer, adipocytes were incubated with dodecanoic acid fluorescent fatty acid analogue (DAA) (QBT™ Fatty acid uptake assay kit, Molecular Devices, Cat. R6138) for 3 hours and washed three times in PBS. Primary AML blasts were then cultured alone or with labelled adipocytes for 24 hours. AML blasts were then removed and fluorescence was subsequently measured by flow cytometry to indicate transfer of fluorescent labelled fatty acid from adipocytes to AML.

Real-time PCR

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Total RNA was extracted from co-cultured BMSC, adipocytes and AMLs and from monoculture of the same using Nucleic Acid PrepStation from Applied Biosystems (Cat. 6100). RNA PCR core kit was used for reverse transcription. SYBR green I dye (Roche, Cat. 04887352001) was used for real-time quantitative PCR using human FABP4 (F: CCACCATAAAGAGAAAACGAG, R: AGTTGCTTGCTAAATCATGG), human GAPDH (F: CTTTTGCGTCGCCAG, R:TTGATGGCAACAATATCCAC), human CPT1 (F:TGGATCTGCTGTATATCCTTC, R: AATTGGTTTGATTTCCTCCC), Mouse FABP4 (F: GTAAATGGGGATTTGGTCAC, R: TATGATGCTCTTCACCTTCC). Mouse GAPDH (F: AAGGTCATCCCAGAGCTGAA, R: CTGCTTCACCACCTTCTTGA) primers.

Western immunoblotting

Western analyses following SDS-PAGE were carried out as described. Whole cell lysates were extracted from AML/adipocyte co-culture and monocultures by using radioimmunoprecipitation assay buffer (RIPA) containing 1% NP-40 (Sigma-Aldrich, Gillingham, UK), 50 mmol/L Tris, 10% glycerol, 0.02% NaN3, 150 mmol/L NaCl, and a cocktail of phosphatase and protease inhibitors (Sigma-Aldrich, Gillingham, UK). SDS-Page was performed as previously described. [17,19]

Bioinformatic analysis

Publicly available RNA sequencing data were downloaded for a panel of 43 AML patients, which comprised 22 AML samples obtained from peripheral blood and 21 AML samples obtained from bone marrow aspirate and for 17 non-malignant CD34+ from 17 non-pooled individuals (Gene Expression Omnibus Accession ID: GSE49642 and GSE48846).[20] Data were available as Reads Per Kilobase per Million mapped reads (RPKM). RPKM data for FABP4 were extracted and processed further by first replacing zero-valued entries with one followed by logarithmic transformation to the base 2.[21] FABP4 RPKM values for blood, bone marrow samples and non-malignant CD34+ cells were compared with a Wilcoxon rank-sum test.

Clonogeneic methylcellulose assay

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Primary AML blasts from adipocyte co-culture and monoculture were plated in methylcellulose medium (R&D Systems) at a density of 1 x 10^4 in all experiments. Colonies were visualised and counted after 14 days.

Fatty acid oxidation

β-oxidation of fatty acids was assessed using the Seahorse XFp Analyzer and the Seahorse XF Palmitate-BSA FAO Substrate kit (Agilent Seahorse Bioscience) according to manufacturer's specifications. Briefly, AML blasts or non-malignant CD34+ cells were cultured with or without adipocytes and then removed from co-culture and placed in substrate limited media (supplemented with 0.5mM glucose, 0.5mM carnitine, 1mM glutamine and 1% FBS) for 4 hours before assaying. AML blasts were then plated in poly-D-lysine (Sigma) coated assay wells at a density of $2x10^5$ per well in base media containing 2.5mM glucose, 0.5mM carnitine and 5mM HEPES and adjusted to pH7.4 with 1N NaOH. ETX (40µM) or BSA and Palmitate:BSA where added into the injection ports. The experimental template was designed using Wave software for desktop from Seahorse Bioscience.

Statistical analysis

We used the Mann-Whitney U test to compare results between groups unless otherwise stated in the legends. Results with $P < 0.05$ were considered statistically significant (denoted by *). Results represent the mean ± Standard Deviation of 4 or more independent experiments. For Western blotting, data are representative images of 3 independent experiments. We generated statistics with Graphpad Prism 5 software (Graphpad, San Diego, CA, USA).

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Results

BM Adipocytes support the survival and proliferation of primary AML.

Cellular proliferation is a highly energy-intensive process. Since adipocytes store energy in the form of triglycerides, we hypothesised that adipocytes provide energy in the form of fatty acids (FA) to AML blasts to support rapid growth. Initially, to determine the presence of lipids in AML we examined freshly isolated AML cells for lipid stores using the neutral lipid dye (BODIPY® 493/503) and find that freshly isolated AML cells (CD34+) contain abundant lipids (figure 1A). Figure 1B and 1C shows that lipid stores using the neutral lipid dye are depleted in AML and non-malignant CD34+ cells when cultured in vitro. Supplementary Figure 2 show that AML lose lipid content even when co-cultured with BMSC. As primary AML cells undergo spontaneous apoptosis when cultured in vitro to determine if adipocytes can support the survival of primary AML we cultured primary AML blasts, either alone or in co-culture with adipocytes derived from BM mesenchymal progenitor cells. Figure 1D and Supplementary Figure 3A show that primary AML blasts co-cultured with adipocytes (n=11) are protected from undergoing apoptosis for up to 6 days when compared to AML blasts cultured alone.

Next we wanted to determine if BM adipocytes can support the proliferation of primary AML blasts. Primary AML blasts increase in number when co-cultured with BM adipocytes for 6 days, compared to AML monoculture and AML cultured on BMSC (n=12) (figure 1E). Figure 1F shows that adipocytes also support non-malignant CD34+ cell survival. Supplementary Figure 3B and 3C show that adipocytes support AML proliferation as measured by BrdU incorporation. Supplementary figure 4 shows AML blasts on BMSC and adipocytes from the same patient. In AML blast colony forming cell (CFC) assays, adipocyte co-culture promoted CFC growth when compared to AML mono-culture (Figure 1G). Finally we showed that primary AML blasts from patient samples (n=4) which had been cultured on BM derived adipocytes for 6 days reliably engrafted NSG mice (Figure 1H). The same AML cells cultured in isolation did not engraft NSG mice after 12 weeks. AML were stained for CD33 and CD45 to confirm cell identity.[16,22] We also examined the effect of cytokines on AML survival compared to co-culture with adipocytes. Supplementary Figure 5A shows that there is a significant increase in survival of AML when cultured on adipocytes compared to with a media supplemented with

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cytokines. Supplementary Figure 5B shows that adipocytes support the engraftment of AML blasts compared to AML grown with cytokines. Taken together, these results show that adipocytes maintain AML progenitor cells.

AML blasts induce adipocyte lipolysis.

Our results indicate that adipocytes confer a proliferative advantage to primary AML. We hypothesized that this could be due to transfer of FA from the adipocytes to AML. Therefore, to understand the effect AML cells have on adipocytes we assessed FA and glycerol release. Co-culture of AML with adipocytes increases FA and glycerol release compared to adipocyte and AML alone. (Figure 2A). Moreover, non-malignant CD34+ cells could also induce lipolysis of adipocytes, albeit at a much reduced quantity compared to AML (Figure 2A). Supplementary figure 6A shows that conditioned media from AML can also induce adipocyte lipolysis. Moreover, Figure 2B shows that when we culture AML blasts with the FA, oleate, this supports the survival of AML. Lipolytic activation of adipocytes results from the phosphorylation of hormone sensitive lipase (HSL). Figure 2C shows that AML induced phosphorylated HSL (pHSL) in adipocytes. Conversely, acipomox (which is known to inhibit lipolysis, by inactivation of AMP activated protein kinase and downstream pHSL) inhibited AML proliferation when cultured on adipocytes but not alone or on BMSC (Figure 2D). Supplementary Figure 7 shows that adipocytes have significantly more HSL mRNA expression compared to BMSC and AML. To determine if lipids detected in the tumor cells after co-culture were derived from adipocytes, we cultured primary AML blasts with either adipocytes or BMSC that had previously been loaded with a fluorescent lipid dye (DAA). Supplementary Figure 8 shows loading of DAA into both BMSC and adipocytes. During co-culture we observed that lipids were transferred from adipocytes to AML (Fig. 2E, 2F and Supplementary Figure 9), moreover this transfer was inhibited by acipomox, thus supporting a model in which adipocytes provide lipids to support tumor growth.

FABP4 is important for the transfer of lipids from adipocytes to AML

FABP4 is a carrier protein for FA expressed in adipocytes,[23] and supports the movement of long chain fatty acids generated by lipolysis to the extracellular membrane and beyond.[24,25] Additionally, increased FABP4 has been shown to be

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involved in the transfer of FA from adipocytes to breast and ovarian cancer cells.[12,14] We therefore examined the expression of FABP4 in human adipocytes and BMSC cultured with AML blasts or non-malignant CD34+ cells. RNA expression data shows that FABP4 levels are increased in adipocytes but not BMSC when cultured with AML, moreover non-malignant CD34+ cells had no effect on adipocyte FABP4 mRNA expression (Figure 3A). We also found that AML conditioned media could induced FABP4 up regulation in adipocytes (Supplementary Figure 10A). Next we examined if other genes associated with lipolysis were also induced in adipocytes when cultured with AML. Supplementary Figure 11A shows that ATLG and MGLL mRNA expression are not changed in adipocytes when cultured with AML. In an apparent paradox, when the expression of FABP4 protein was examined, a reduction was observed in adipocytes cultured with AML compared to adipocytes alone (Figure 3B). FABP4 is known to be released from adipocytes as they transport the FA,[26] therefore FABP4 expression in the culture media was measured by a FABP4 specific ELISA. Figure 3C shows that culturing AML with adipocytes induces a release of FABP4 into the culture media. To determine if the FABP4 released by the adipocyte is taken up by the AML we used a recombinant FABP4 tagged to a His sequence. AML were cultured with and without the recombinant FABP4 for 4 hours. Figure 3D shows that no recombinant FABP4 was detected in AML. Next we examined if we could prevent the increase of lipids in AML blast by knockdown of FABP4 in the adipocyte. Figure 3E shows the knockdown FABP4 protein in adipocytes and figure 3F demonstrates that knockdown of adipocyte FABP4 inhibited the transfer of lipids to AML. Furthermore, FABP4 chemical inhibitor (BMS309403) inhibits AML blast survival but had no effect on non-malignant CD34+ cells (Figure 3G). Finally, FABP4 lentiviral knockdown in adipocytes (Figure 3H) inhibits AML blast survival. Together these results demonstrate that FABP4 is critical for the transfer of FA from adipocytes to AML blasts.

As Herroon and colleagues have shown that FABP4 in prostate cancer cells is upregulated in response to culture with adipocytes,[15] we next examined whether FABP4 expression levels were elevated in AML cells extracted from bone marrow as compared to AML cells extracted from peripheral blood or non-malignant CD34+ cells. We found FABP4 expression to be significantly higher in AML bone marrow samples than in AML from peripheral blood or non-malignant CD34+ cells ($P <$

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0.001, Wilcoxon rank-sum test) (Figure 4A). To confirm that this upregulation of FABP4 was a result of AML interacting with adipocytes rather than BMSC we examined FABP4 expression in AML cultured alone, with adipocytes or BMSC. Supplementary figure 12 shows the purity of AML after separation from adipocytes co-culture. Figure 4B shows that FABP4 RNA is upregulated in AML in response to co-culture with adipocytes but not BMSC. We determined whether knockdown of FABP4 in AML cells could reduce survival when cultured on adipocytes. Figure 4C shows that FABP4-KD AML cells have reduced viability when co-cultured with adipocytes compared to co-culture with BMSC. Furthermore knockdown of AML FABP4 inhibited the transfer of lipids from adipocytes to AML (figure 4D).

Next, we sought to determine the functional role of FABP4 in an AML mouse model. To do this we used a retroviral AML transplantation model where Hoxa9 and Meis1 are used to transform myeloid progenitor cells as described by Kroon et al (1998).[27] Initially we determined if Hoxa9/Meis1-expressing cells could proliferate on adipocytes compared to BMSC or monoculture or monoculture without cytokine supplements. Figure 4E shows that Hoxa9/Meis1-expressing cells proliferate on adipocytes and BMSC. Next we knocked down FABP4 in Hoxa9/Meis1-expressing cells (figure 4F) and found that FABP4 KD reduced AML survival when cultured with adipocytes compared to media supplemented with cytokines or BMSC (Figure 4G). Moreover, knockdown of FABP4 in Hoxa9/Meis1-expressing cells also reduces FA uptake, quantified by DAA fluorescence, when tumor cells were cultured on adipocytes (Figure 4H). Finally we show in vivo that FABP4-KD in Hoxa9/Meis1 expressing blasts significantly increases animal survival (Figure 4I). Taken together these results demonstrate that FABP4 in AML is essential for FA transport and tumor proliferation in the BM microenvironment.

Co-culture of AML with adipocytes activates β-oxidation in AML blasts

In order to study the reliance of AML blasts on adipocytes for their energy we first measured the cellular oxygen consumption rate (OCR) in AML blasts to determine levels of fatty acid oxidation (FAO). To confirm that AML blasts can use oxidation of exogenous fatty acid we assessed FAO by monitoring OCR upon addition of palmitate (FAO substrate) followed by addition of etomoxir (FAO inhibitor - ETX). Figure 5A shows that primary AML have increased OCR in the presence of palmitate

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which is inhibited by ETX this is compared to non-malignant CD34+ cells. Oxidation of exogenous fatty acid can be measured by examining OCR of AML blasts and non-malignant CD34+ cells in response to ETX. Figure 5B and Supplementary Figure 13 shows that AML blasts cultured with adipocytes have increased FAO compared to AML blasts cultured alone or with BMSC and non-malignant CD34+ cells. In addition, we observed that AML blasts cultured on adipocytes had greater basal OCR compared to AML cultured alone or on BMSC (Figure 5C). OCR was reduced in AML co-cultured on adipocytes compared to BMSC with FABP4 knocked down compared to control KD cells (Figure 5D). Moreover, because of the nature of the OCR experiment in which AML were serum starved for 4 h prior to being loaded onto the Seahorse Bioanalyser we show that AML FABP4 mRNA levels were stable compared to non serum starved AML cells (Supplementary Figure 10B). In addition, the FAO inhibitor ETX reduced AML blast survival when grown on adipocytes but not on BMSC (figure 5E).

ETX is a selective inhibitor of carnitine palmitoyltransferase-1 (CPT1A), which transports fatty acyl chains from the cytosol into the mitochondria, a process essential for the production of ATP from fatty acid oxidation.[28] We therefore examined the expression of various genes including CPT1A in AML when cultured with adipocytes. Supplementary Figure 11B shows that CPT1A, CPT2 and ACADL are all up-regulated in AML when cultured with adipocytes. Accordingly, we knocked-down CPT1A in AML blasts. Figure 5F shows a reduction in CPT1A mRNA and protein in human AML cells after infection with CPT1A shRNA lentivirus. Following successful knockdown of CPT1A in AML cells we found that CPT1A-KD AML cells have reduced viability when cultured with adipocytes compared to cells cultured on BMSC (Figure 5G). Finally, we tested the effect of CPT1A-KD in an AML patient derived xenograft model. Figure 5H shows that NSG mice engrafted with CPT1A-KD AML blasts from 2 patient samples have increased survival compared to control-KD NSG mice (n=4). Supplementary Figure 14 shows the bone marrow and spleen engraftment data for Figure 5H.

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Discussion

Here we report that bone marrow adipocytes support the survival and proliferation of AML blasts. We find that AML induces lipolysis of triglyceride stored within BM derived adipocytes. Subsequently the FA released by triglyceride lipolysis within adipocytes is transported out of the adipocyte in a process dependent on the chaperone protein FABP4. Proximity to adipocytes also results in up-regulation of the same chaperone protein, FABP4, within the blasts, which is then used to transport adipocyte derived FA to the mitochondria within the tumor cells. The AML mitochondria use the FA as a substrate for B-oxidation generating the energy required for leukemic growth and proliferation.

AML arises from malignant transformation and proliferation of hematopoietic progenitor cells in the BM microenvironment. AML is co-located with BM adipose tissue (MAT), which is a biologically active energy storage and endocrine organ and accounts for approximately 70% of bone marrow volume in adult humans.[8,29] Like the prevalence of AML, BM adipocytes are known to increase with age and furthermore are not merely passive occupants of the bone marrow but are now appreciated to be actively involved in processes linked to bone metabolism, osteoporosis, inflammation and regulation of the hematopoietic niche.[30] Moreover, adipocytes have been shown to support the proliferation of solid tumor cells in studies of breast, ovarian and prostate cancer metastases.[12,14,15] Here we describe the pro-tumoral function of bone marrow derived adipocytes in AML.

We report that leukemia cells cause functional changes in non-malignant adipocytes, which ultimately leads to the transfer of free fatty acids from adipocytes to the AML blast. This is a FABP4 dependent process. FABP4 has intracellular functions in adipocytes but is also known to be actively secreted by adipocytes where it can exert specific biological functions in tissues other than it's origin.[31] Co-culture of AML and adipocytes results in transcriptional up-regulation of FABP4, in both in the adipocyte and the leukemic blast. Curiously however, we found that despite this transcriptional increase of FABP4 in the adipocytes, the intra-adipocyte protein level of FABP4 is decreased upon co-culture with AML. This led us to hypothesise that either (1) adipocyte FABP4 rapidly chaperones the FA from the adipocyte into the microenvironment (2) adipocyte FABP4 rapidly chaperones the FA from the

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adipocyte into the AML cells or (3) excess intracellular FABP4 is simply degraded within the adipocyte. To consider this further we investigated the levels of FABP4 in the media of AML adipocyte co-cultures and confirmed FABP4 was released into the medium. In models of prostate cancer recombinant FABP4 is taken up by the malignant cells.[32] In our studies we also conducted experiments using recombinant FABP4 to determine if AML blasts actively take up this protein. We showed that no recombinant FABP4 was detected in AML after 4 hours of culture. These data suggest that unlike prostate cancer FA/FABP4 is not directly transferred from adipocyte to the AML cell but may be involved in an extracellular-to-membrane transport whereby it is degraded after functionality outside of the cell. Further studies would need to be conducted to confirm this hypothesis.

Maintenance of AML appears to depend on both the adipocyte and the leukemic blast being able to generate FABP4. Pharmacological inhibition or lentiviral-knockdown of FABP4 in the adipocytes showed a significant inhibitory effect on AML survival in co-culture experiments. Fatty acid binding proteins bind free FA, which otherwise in their free form are relatively insoluble and potentially toxic. Furthermore, FABP4 binds and donates its fatty acid ligand via collisional interactions with membranes and is actively secreted from adipocytes.[31,33] We suggest that the reduced AML survival on FABP4 KD adipocytes is due to the inability of FA to be secreted from the adipocytes in the absence of FABP4 protein. In addition we observed that FABP4 knock down in the AML cells improved survival in our in-vivo models (although was unable to ultimately prevent leukemic engraftment). We also compared FABP4 transcript levels between AML blasts taken from the bone marrow and the peripheral blood, and found that FABP4 was enriched in AML cells collected from the adipose rich environment of the BM, indicating that FABP4 expression in AML blasts requires an external BM derived signal. Taken together we propose that in AML, FA is transported in the BM adipocyte by adipocyte derived FABP4, then secreted and taken up by the AML and transported within the AML by AML derived FABP4. We conclude that delivery and intracellular transport of FA to and in AML blasts is dependent on the presence of functional bone marrow adipocytes.

When we compare our in vitro FABP4 knock down experiments in primary human AML cells and HoxA9/Meis1 cells, the effect in the HoxA9/Meis1 experiments is

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comparatively modest. However, in contrast when FABP4 was knocked down in HoxA9/Meis1 cells, which were then injected into mice, we observed a more demonstrable anti-tumor effect. In addition we also found that our AML blasts maintained on adipocytes appeared to engraft (range 4-6 weeks) earlier than expected when compared to other reports in which primary AML blasts had not been previously cultured on adipocytes.[34-36] We have not investigated this observation formally but postulate that adipocytes may support the maintenance of primary leukemic progenitor cells. Overall, we suggest that our in-vivo model more closely recapitulates the complexity of a tumor growing within a patient than the in-vitro assays and also propose that adipocytes may improve efficiency of ex-vivo culture systems for primary human AML.

Blocking lipolysis using Acipomox or inhibiting transfer of free fatty acids using a FABP4 inhibitor only decreased AML viability between 40–60%. This could in part be due to the efficiency of the drug to inhibit its target. Alternatively perhaps this implies that adipocytes contribute other pro-AML survival factors in addition to free fatty acids. Adipocytes have been shown to secrete pro-inflammatory cytokines in the presence of malignant cells which may have an impact on tumor cell migration and survival.[37] This has specifically been shown in the context of leukemias by Ye and colleagues, who report that bone marrow and gonadal adipose tissues show high levels of tumor associated pro-inflammatory cytokine secretion, several of which had previously been implicated in tumor cell migration.[37-39] Therefore, it is likely that BM adipocytes provide more than just FA to AML to facilitate the proliferative capacity of this disease.

We found that the OCR of AML cells increased in adipocyte co-culture experiments and that inhibition of CPT1A in the leukemic cells then significantly reduced AML OCR and survival. CPT1A is essential for the transfer of FA to the inner mitochondrial membrane for acetyl-CoA generation via B-oxidation,[40] and these data indicate that the BM adipocytes are a source for the FA necessary for AML metabolism. Our observations are also consistent with the work of others who have previously reported the importance of β-oxidation in AML survival and proliferation.[41,42] Conversely, in control experiments, non-malignant CD34 positive cells did not appear to significantly decrease their OCR upon treatment with the

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CPT1A inhibitor, etomoxir, indicating that this FA oxidation signature is particularly associated with leukemic progenitors.

In summary, although adipocytes in the non-malignant setting appear to be negative regulators of hematopoietic microenvironment.[43] in the context of disease, adipocytes like other cells, can undergo distinct pathologic changes.[44] Here we report that in the setting of AML, BM derived adipocytes support tumor proliferation and survival. Specifically, AML blasts modulate intra-cellular adipocyte metabolism into a lipolytic state resulting in the release of FA into the microenvironment. Ultimately, this free fatty acid is metabolically beneficial to the leukemia. Accordingly, we hypothesise that identification of this pro-tumoral interaction will open up potential novel therapeutic strategies in the treatment of human AML.

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Authorship contributions

M.S.S., T.O, K.M.B. and S.A.R. designed the research; M.S.S., S.M., L.Z. and A.A.A. performed the research; M.S.S., S.D.R., S.M., C.M., R.P. carried out in vivo work; D.R.E., M.F., J.T., M.L. and K.M.B. provided essential reagents and knowledge. J.A.W. performed the bioinformatics. M.S.S., K.M.B., T.O. and S.A.R. wrote the paper.

Conflict of interest

SAR reports research funding from Infinity Pharmaceuticals

All other authors declare no competing financial interests

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Table 1. AML patient sample information used in this study. This table defines the nature of the AML disease including WHO diagnosis and cytogenetics.

Number	Age	Sex	WHO diagnosis	Cytogenetics	% blasts
AML#1	69	M	AML NOS	N/A	60
AML#2	66	F	AML without maturation	Trisomy 9	90
AML#3	41	F	AML with t(6;9)(p23;q34); DEK-NUP214	t(6;9)	95
AML#4	62	M	AML with maturation	complex	55
AML#5	70	M	AML with maturation	Normal	80
AML#6	70	M	AML without maturation	Complex	95
AML#7	91	F	AML NOS	N/A	60
AML#8	55	F	AML	Not avaiable	70
AML#9	59	F	AML with t(8;21)(q22;q22) RUNX1-RUNX1T1	t(8;21)	80
AML#10	78	F	AML without maturation	Normal	95
AML#11	58	F	AML with maturation	Normal	80
AML#12	65	M	AML with maturation	Trisomy 13	30
AML#13	73	F	AML without maturation	Normal	95
AML#14	49	M	AML with myelodysplasia related changes	N/A	55
AML#15	64	F	AML with myelodysplasia related changes	Normal	45
AML#16	65	M	AML with minimal differentiation	Normal	95
AML#17	23	F	AML without maturation	t(5;12)	95
AML#18	37	M	AML without maturation	Normal	90
AML#19	59	M	AML with t(8;21)(q22;q22) RUNX1-RUNX1T1	t(8;21)	60
AML#20	65	M	AML with maturation	Not available	70
AML#21	68	M	Therapy related AML	Not available	40
AML#22	63	M	AML with myelodysplasia related changes	Not available	30
AML#23	76	M	AML without maturation	Not available	95
AML#24	75	M	AML with maturation	Complex	55
AML#25	88	M	AML with maturation	Trisomy 8	30
AML#26	35	M	AML without maturation	46 XY	80
AML#27	72	M	AML with myelodysplasia related changes	46,XY,isochrome (17)(q10)	90

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Figures Legends

Figure 1. BM adipocytes support the survival and proliferation of primary AML. (A) Immunofluorescence of primary AML blasts stained for CD34+ (red) and neutral lipids (green) using BODIPY® 493/503. All images are representative of 6 AML patient samples. Scale bar = 10 micron (B) Freshly isolated AML, AML cultured for 1 day and AML cultured for 2 days stained with the neutral lipid BODIPY® 493/503 dye and analysed by flow cytometry. Data represented as mean ± standard deviation. (C) Freshly isolated non-malignant CD34+ cells and freshly isolated AML samples stained with the neutral lipid BODIPY® 493/503 dye and analysed by flow cytometry. (D) AML patients samples in monoculture and co-cultured with bone marrow-derived adipocytes for 6 days and then stained with PI/Annexin V. The line through the data indicates the median. (E) AML blasts incubated alone or with adipocytes or BMSC for 6 days and AML blasts counted using flow cytometry and trypan blue exclusion (n=12). The line through the data indicates the median (F) Non-malignant CD34+ cells cultured alone or in co-culture with BMSC or adipocytes and CD34+ cell counted using flow cytometry and trypan blue exclusion (n=5). The line through the data indicates the median (G) AML blasts from 3 different patients were cultured alone or with adipocytes or BMSC for 6 days and then placed in a colony forming cell (CFC) assay for 15 days. Colonies were then counted. Data represented as mean ± standard deviation (H) $2x10^6$ primary AML cells (4 individual patient AML) cultured on BM adipocytes or cultured alone and then $2x10^6$ viable cells were injected into NSG mice. Engraftment was measured using human CD33 and human CD45. Shown in the flow figure are the characteristics of AML#12 engraftment into BM and spleen. In the dot plot each AML engraftment into NSG mice is shown for bone marrow and spleen, the engraftment of the AML cultured alone is shown by a shaded circle. The line through the data indicates the median.

Figure 2. AML blasts induce adipocyte lipolysis. (A) Primary AML blasts or non-malignant CD34+ cells were cultured alone or in co-culture with adipocytes for 24h, media was removed and used to detect free fatty acid and glycerol. Data represented as mean ± standard deviation (B) AML blasts incubated with media supplemented with BSA or media supplemented with 100µM of oleate-BSA conjugate for 2 days and AML blasts counted using flow cytometry and trypan blue

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exclusion (n=4). The line through the data indicates the median (C) Immunoblot for pHSL from adipocytes cultured with and without AML blasts (n=4). Blots were repobed for total HSL and β-actin to show equal sample loading (D) Primary AML blasts in monoculture or cultured with adipocytes or BMSC with and without treatment with acipomox (APX) (10 μM) for 72 hours. AML blasts were counted using flow cytometry and trypan blue exclusion (n=4). The Mann-Whitney U test was used to determine statistical significance between treatment groups. Data represented as mean ± standard deviation (E) and (F) AML blasts cultured on adipocytes (with and without acipomox (10 μM)) or BMSC that had been pre-incubated with fluorescent dodecanoic acid analogue (DDA) for 24 h (n=4). Blasts were analysed for uptake of the fluorescent dodecanoic acid analogue by flow cytometry. Data represented as mean ± standard deviation. In panel (E) the red line is AML cultured on BMSC; the black, AML cultured on adipocytes and the blue, AML cultured on adipocytes treated with acipomox.

Figure 3. FABP4 controls the transfer of lipids from adipocytes to AML. (A) AML blasts were cultured alone or with adipocytes or BMSC for 48h and then the adipocytes and BMSC were assessed for FABP4 mRNA expression using real-time PCR (n=6). non-malignant CD34+ cells were co-cultured alone or with adipocytes and FABP4 mRNA expression was measured. Data represented as mean ± standard deviation (B) Immunoblot for FABP4 from adipocytes cultured with and without AML blasts. Blots were reprobed for β-actin to show equal sample loading. (C) ELISA to detect FABP4 in media from adipocytes cultured alone or with AML blasts. Data represented as mean ± standard deviation. (D) AML blasts were cultured alone or with the addition of 2ug/ml of recombinant FABP4 (his-tagged) for 4 h. Immunoblots were performed for FABP4 and His. Blots were reprobed for β-actin to show equal sample loading. (E) Adipocytes were infected with FABP4-targeted shRNA or control shRNA lentivirus and after 72h analysed for FABP4 protein expression using Western Blotting. Blots were reprobed for β-actin to show equal sample loading. (F) Adipocytes were infected with FABP4-targeted shRNA or control shRNA lentivirus and after 72h preloaded with dodecanoic acid fluorescent fatty acid analogue and incubated with AML for 24h. AML blasts were analysed for fluorescence using flow cytometry (n=4). (G) Primary AML blasts or non-malignant CD34+ cells were cultured alone or co-cultured with adipocytes or BMSC with and

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without treatment with FABP4 inhibitor for 72h. AML blasts were counted using flow cytometry and trypan blue exclusion (n=4). Data represented as mean ± standard deviation. (H) Adipocytes were infected with FABP4 targeted shRNA or control shRNA lentivirus and after 72h were incubated with AML for 72h. AML blasts and non-malignant CD34+ cells were counted using flow cytometry and trypan blue exclusion (n=4). Data represented as mean ± standard deviation.

Figure 4. AML derived FABP4 is crucial for blast survival in vivo. (A) FABP4 gene expression (expressed in log2 RPKM values) was obtained from GSE49642 and GSE48846 for non-malignant CD34+ cells, 22 blood AML and 21 bone marrow AML patient samples. P-value was obtained by Wilcoxon rank-sum test. Middle band denotes the median value with lower and upper bands denoting the first and third quartiles, respectively. (B) AML blasts were cultured alone or with adipocytes or BMSC for 48h before the AML were assessed for FABP4 mRNA expression using real-time PCR (n=6). Data represented as mean ± standard deviation. (C) Primary AML blasts were infected with FABP4 shRNA1 and shRNA2 and control shRNA and after 96h were subsequently cultured on adipocytes or BMSC for a further 72h. AML blasts counted using flow cytometry and trypan blue exclusion (n=4). Data represented as mean ± standard deviation. (D) Primary AML were infected with FABP4-targeted shRNA1 or control shRNA lentivirus and after 96h were cultured with adipocytes pre-loaded with dodecanoic acid fluorescent fatty acid analogue for 24h. AML blasts were analysed for fluorescence using flow cytometry. (E) Hoxa9/Meis1 transformed cells ($1x10^5$/ml) were cultured normal media or normal media with IL-3, IL-6 and SCF supplemented or co-cultured on BMSC with normal media or on adipocytes with normal media. Hoxa9/Meis1 expressing cells were counted using flow cytometry and trypan blue exclusion (n=4). Data represented as mean ± standard deviation. (F) Hoxa9/Meis1 expressing cells were infected with mouse FABP4 shRNA or control shRNA and after 72h analysed for FABP4 protein expression using Western Blotting. Blots were reprobed for β-actin to show equal sample loading. (G) Hoxa9/Meis1 expressing cells were infected with FABP4 targeted shRNA or control shRNA and after 72h incubated either alone, with cytokines, with BMSC or with adipocytes. Hoxa9/Meis1 expressing cells counted using flow cytometry and trypan blue exclusion (n=4). Data represented as mean ± standard deviation. (H) Hoxa9/Meis1 expressing cells were infected with FABP4

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targeted shRNA or control shRNA lentivirus and after 72h incubated for 24h with adipocytes preloaded with dodecanoic acid fluorescent fatty acid analogue. Hoxa9/Meis1 expressing cells were analysed for fluorescence using flow cytometry (n=4). (I) Kaplan-Meier survival curves for C57BL/6 mice injected with Hoxa9/Meis1 FABP4-KD cells or Hoxa9/Meis1 AML control-KD cells.

Figure 5. Co-culture of AML with adipocytes activates β-oxidation in AML cells. (A) Primary AML blasts or non-malignant CD34+ cells were cultured on adipocytes for 3 days and then starved for 4h before measuring OCR (pMoles/min) using the Seahorse XFp Analyzer, at baseline and then after injection of palmitate (18 mins) and ETX (36 mins). Circles represent ETX (40μM) treatment and squares represent no ETX treatment. (B) Primary AML blasts or non-malignant CD34+ cells were cultured alone for 3 days and AML blasts cultured on adipocytes for 3 days and then starved for 4h. AML blasts were then treated with ETX (40μM) and OCR was measured as above (n=4). Data represented as mean ± standard deviation. (C) Primary AML blasts cultured alone for 3 days and AML blasts cultured on adipocytes or BMSC for 3 days and then starved for 4h. Data represented as mean ± standard deviation. (D) AML were infected with FABP4 targeted shRNA or control shRNA lentivirus and after 72h incubated with adipocytes or BMSC for 24h and OCR was measured in the AML (n=4). Data represented as mean ± standard deviation. (E) Primary AML blasts were in monoculture or co-cultured on adipocytes or BMSC with and without treatment with ETX for 72 hours. AML blasts were counted using flow cytometry and trypan blue exclusion (n=4). Data represented as mean ± standard deviation. (F) AML blasts were infected with CPT1A shRNA or control shRNA lentivirus and after 72 hours analysed for CPT1A mRNA and protein expression using RT-PCR and Western Blotting. Blots were reprobed for β-actin to show equal sample loading. Data represented as mean ± standard deviation. (G) AML were infected with CPT1A shRNA or control shRNA lentivirus and then co-cultured on BMSC or adipocytes. AML blasts were counted using flow cytometry and trypan blue exclusion (n=4). Data represented as mean ± standard deviation. (H) 2 Primary AML samples were infected with CPT1A shRNA or control shRNA lentivirus and after 96 hours were then grown on adipocytes for 48 hours and subsequently $2x10^6$ primary AML cells (n=4) were i.v. injected into NSG mice. Survival of the NSG mice is

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represented by a Kaplan–Meier plot. P=0.025 for mice injected with AML CPT1-KD compared to AML control-KD mice.

Figure 1

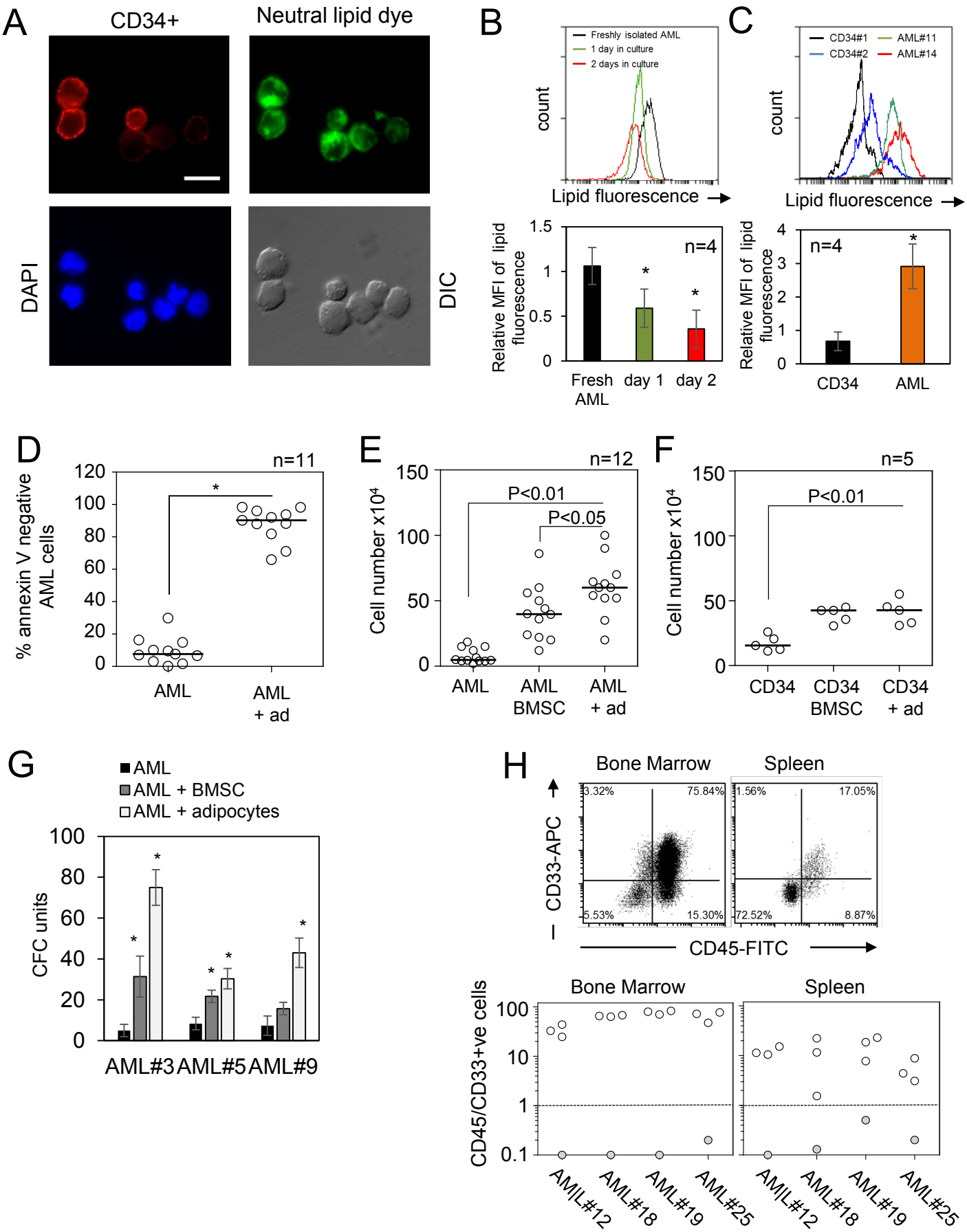

A
CD34+
Neutral lipid dye
DAPI
DIC
B
Freshly isolated AML
1 day in culture
2 days in culture
count
Lipid fluorescence
Relative MFI of lipid fluorescence
n=4
Fresh AML
day 1
day 2
C
CD34#1
CD34#2
AML#11
AML#14
count
Lipid fluorescence
Relative MFI of lipid fluorescence
n=4
CD34
AML
D
% annexin V negative AML cells
n=11
AML
AML + ad
E
Cell number x10⁴
n=12
P<0.01
P<0.05
AML
AML BMSC
AML + ad
F
Cell number x10⁴
n=5
P<0.01
CD34
CD34 BMSC
CD34 + ad
G
AML
AML + BMSC
AML + adipocytes
CFC units
AML#3
AML#5
AML#9
H
Bone Marrow
Spleen
CD33-APC
CD45-FITC
3.32%
75.84%
5.53%
15.30%
1.56%
17.05%
72.52%
8.87%
CD45/CD33+ve cells
AML#12
AML#18
AML#19
AML#25

Figure 2

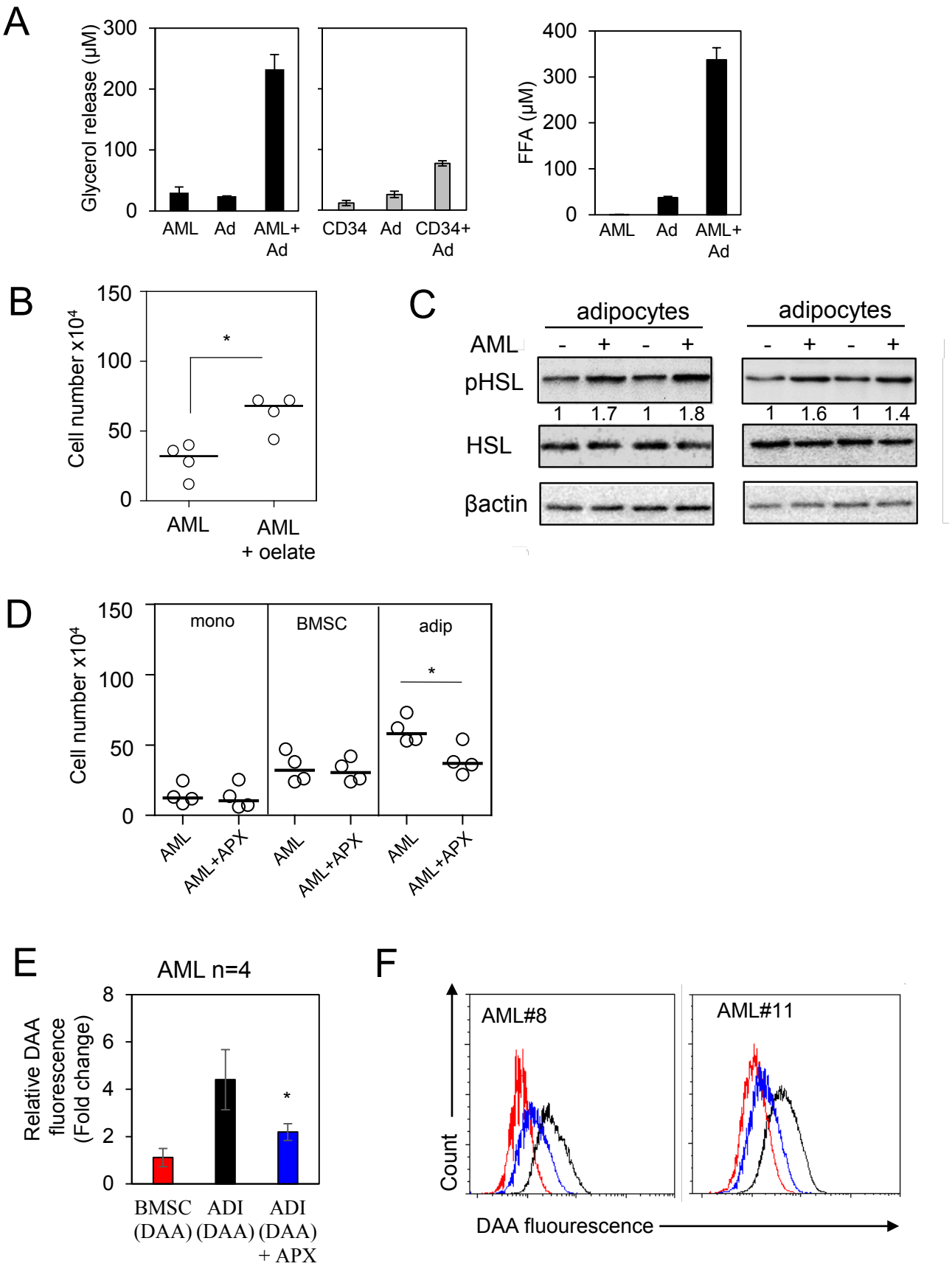

A
Glycerol release (µM)
300
200
100
0
AML
Ad
AML+ Ad
CD34
Ad
CD34+ Ad
FFA (µM)
400
300
200
100
0
AML
Ad
AML+ Ad
B
Cell number x10⁴
150
100
50
0
*
AML
AML + oelate
C
adipocytes
adipocytes
AML
- + - +
- + - +
pHSL
1 1.7 1 1.8
1 1.6 1 1.4
HSL
βactin
D
Cell number x10⁴
150
100
50
0
mono
BMSC
adip
*
AML
AML+APX
AML
AML+APX
AML
AML+APX
E
AML n=4
Relative DAA fluorescence (Fold change)
8
6
4
2
0
*
BMSC (DAA)
ADI (DAA)
ADI (DAA) + APX
F
AML#8
AML#11
Count
DAA fluourescence

Figure 3

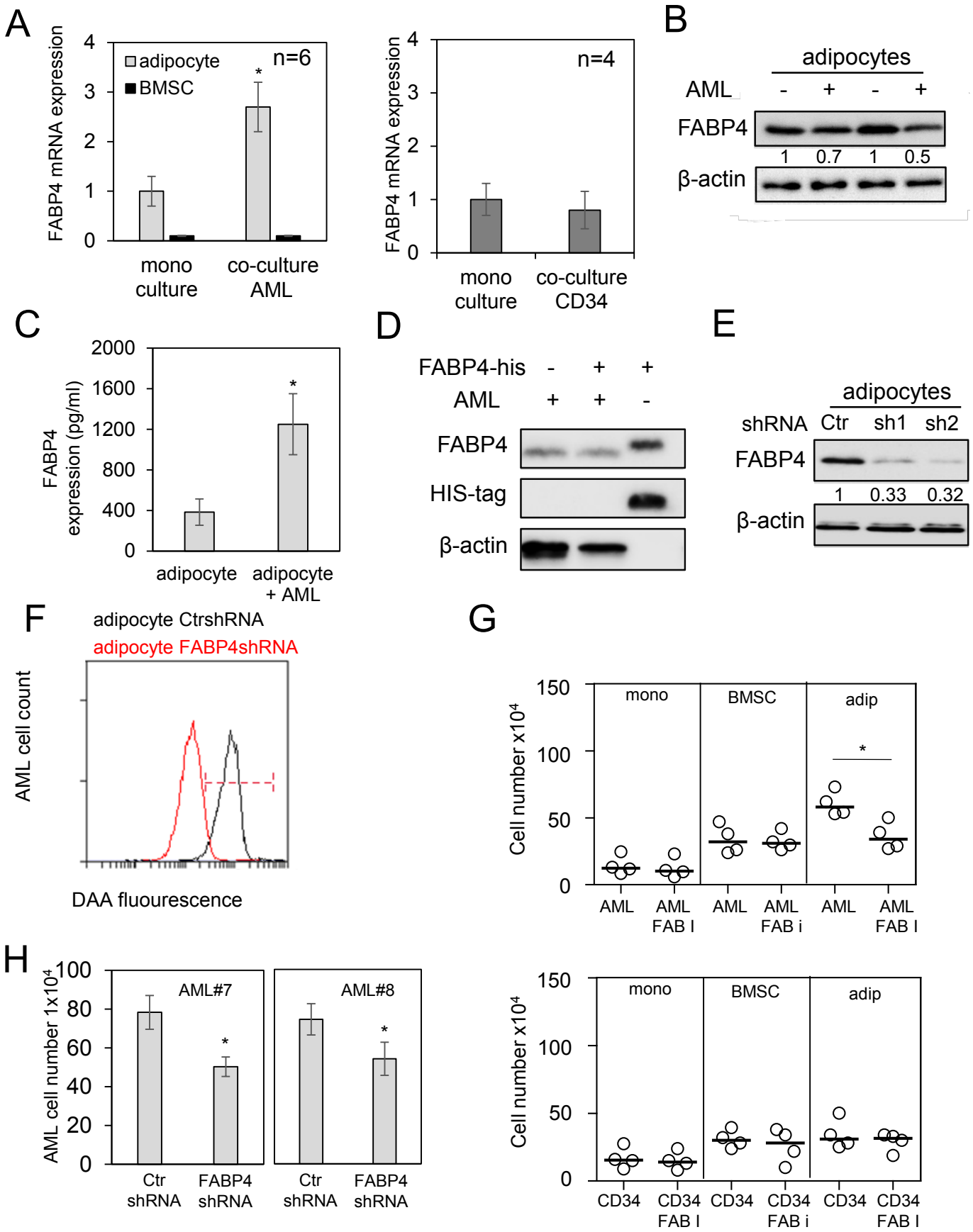

A
FABP4 mRNA expression
adipocyte
BMSC
n=6
mono culture
co-culture AML
n=4
mono culture
co-culture CD34
B
adipocytes
AML - + - +
FABP4
1 0.7 1 0.5
β-actin
C
FABP4 expression (pg/ml)
adipocyte
adipocyte + AML
D
FABP4-his - + +
AML + + -
FABP4
HIS-tag
β-actin
E
adipocytes
shRNA Ctr sh1 sh2
FABP4
1 0.33 0.32
β-actin
F
adipocyte CtrshRNA
adipocyte FABP4shRNA
AML cell count
DAA fluorescence
G
Cell number x10⁴
mono
BMSC
adip
AML
AML FAB I
AML FAB i
H
AML cell number 1x10⁴
AML#7
AML#8
Ctr shRNA
FABP4 shRNA
CD34
CD34 FAB I
CD34 FAB i

Figure 4

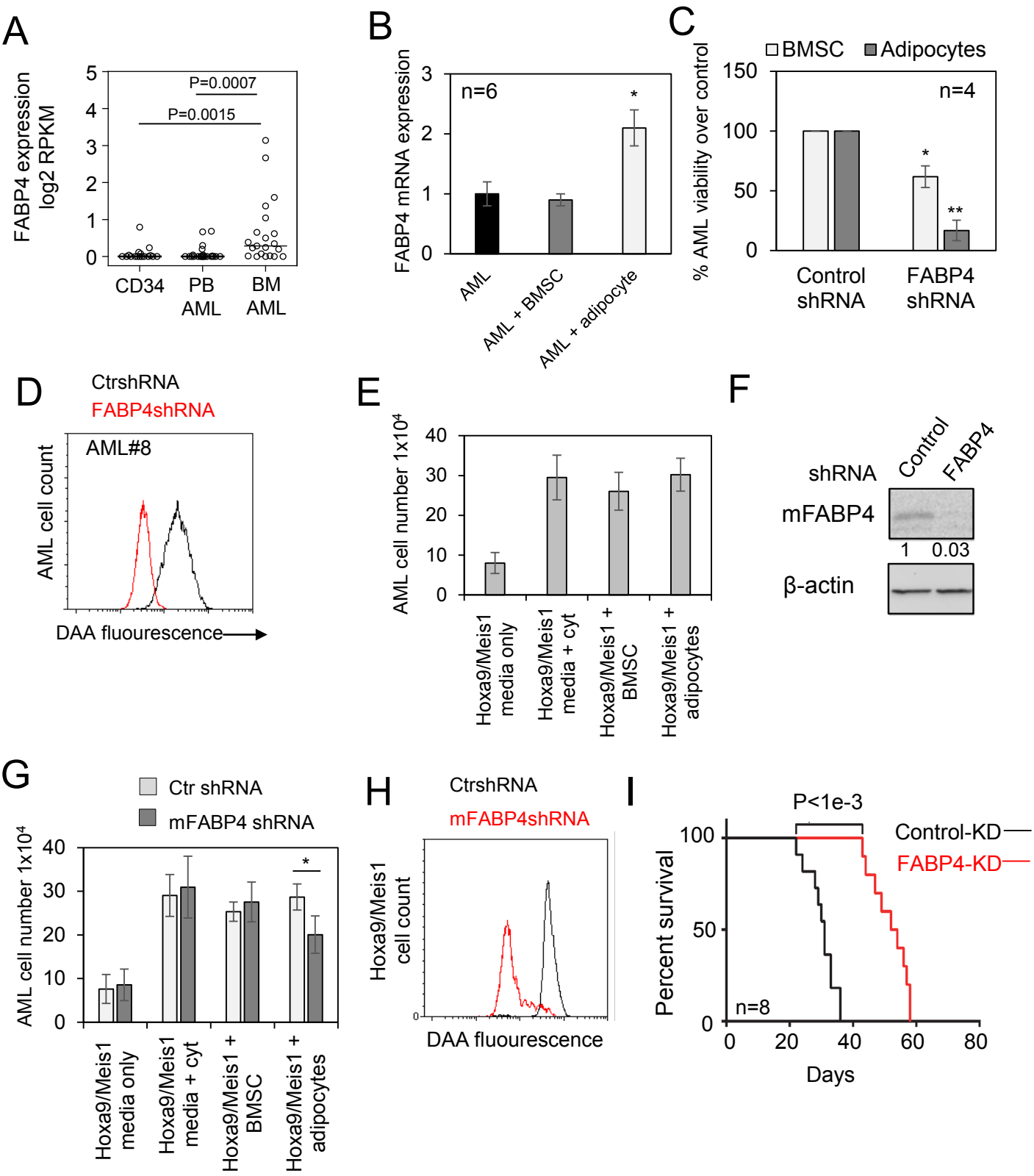
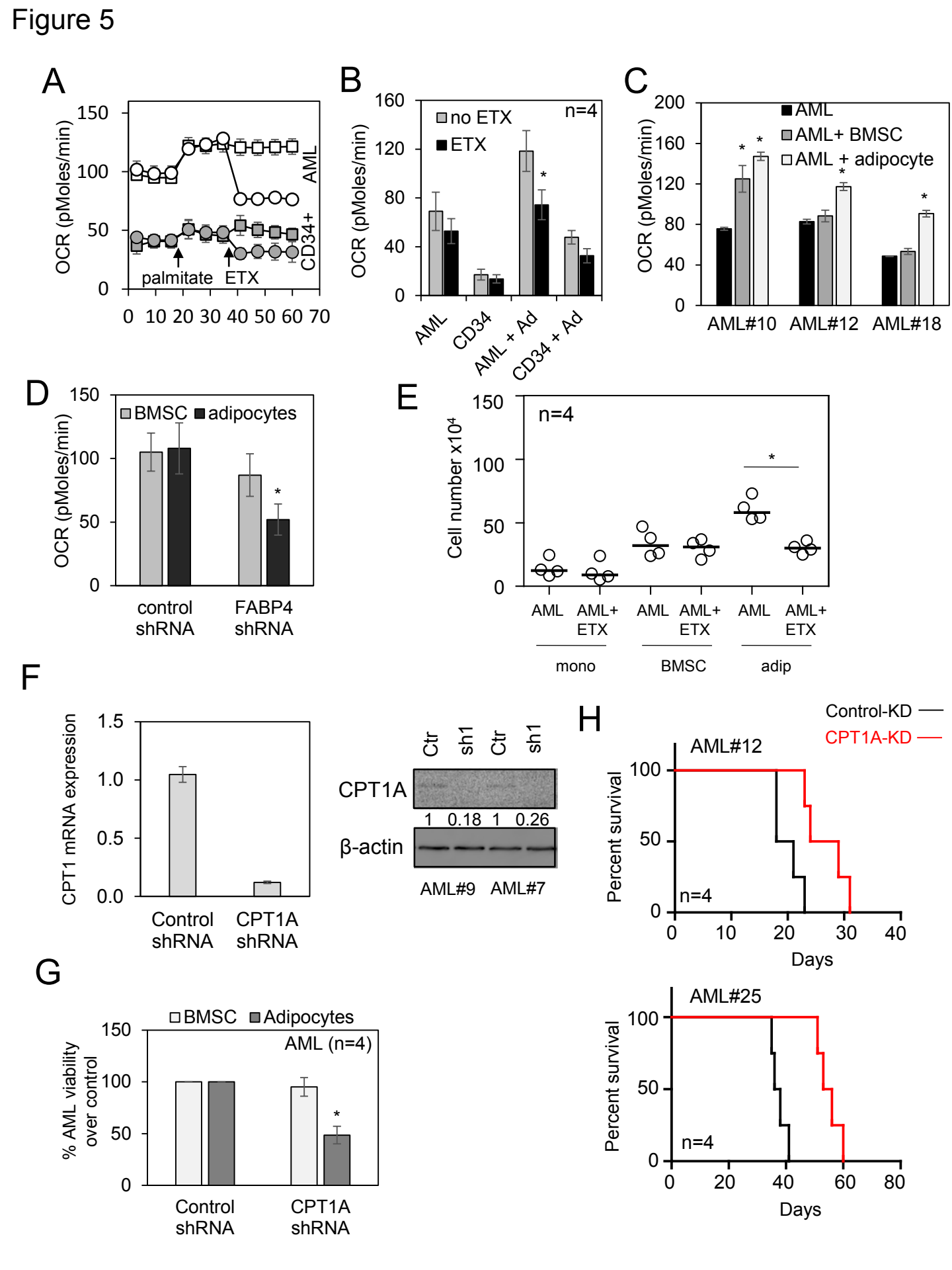

A
FABP4 expression log2 RPKM
P=0.0007
P=0.0015
CD34
PB AML
BM AML
B
FABP4 mRNA expression
n=6
AML
AML + BMSC
AML + adipocyte
C
BMSC
Adipocytes
% AML viability over control
n=4
Control shRNA
FABP4 shRNA
D
CtrshRNA
FABP4shRNA
AML#8
AML cell count
DAA fluourescence
E
AML cell number 1x10^4
Hoxa9/Meis1 media only
Hoxa9/Meis1 media + cyt
Hoxa9/Meis1 + BMSC
Hoxa9/Meis1 + adipocytes
F
shRNA
Control
FABP4
mFABP4
1
0.03
β-actin
G
Ctr shRNA
mFABP4 shRNA
AML cell number 1x10^4
Hoxa9/Meis1 media only
Hoxa9/Meis1 media + cyt
Hoxa9/Meis1 + BMSC
Hoxa9/Meis1 + adipocytes
H
CtrshRNA
mFABP4shRNA
Hoxa9/Meis1 cell count
DAA fluourescence
I
P<1e-3
Control-KD
FABP4-KD
Percent survival
n=8
Days

Figure 5


A
OCR (pMoles/min)
150
100
50
0
AML
CD34+
palmitate
ETX
0 10 20 30 40 50 60 70
B
OCR (pMoles/min)
160
120
80
40
0
no ETX
ETX
n=4
AML
CD34
AML + Ad
CD34 + Ad
C
OCR (pMoles/min)
200
160
120
80
40
0
AML
AML+ BMSC
AML + adipocyte
AML#10
AML#12
AML#18
D
OCR (pMoles/min)
150
100
50
0
BMSC
adipocytes
control shRNA
FABP4 shRNA
E
Cell number x10⁴
150
100
50
0
n=4
AML
AML+ ETX
mono
BMSC
adip
F
CPT1 mRNA expression
1.5
1.0
0.5
0.0
Control shRNA
CPT1A shRNA
Ctr
sh1
CPT1A
β-actin
1 0.18 1 0.26
AML#9
AML#7
G
% AML viability over control
150
100
50
0
BMSC
Adipocytes
AML (n=4)
Control shRNA
CPT1A shRNA
H
Control-KD
CPT1A-KD
AML#12
Percent survival
100
50
0
n=4
0 10 20 30 40
Days
AML#25
Percent survival
100
50
0
n=4
0 20 40 60 80
Days

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Leukemic blasts program bone marrow adipocytes to generate a pro-tumoral microenvironment

Manar S. Shafat, Thomas Oellerich, Sebastian Mohr, Stephen D. Robinson, Dylan R. Edwards, Christopher R. Marlein, Rachel E. Piddock, Matthew Fenech, Lyubov Zaitseva, Amina Abdul-Aziz, Jeremy Turner, Johnathan A. Watkins, Matthew Lawes, Kristian M. Bowles and Stuart A. Rushworth

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