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Stromal CYR61 Confers Resistance to Mitoxantrone via Spleen Tyrosine Kinase Activation in Human Acute Myeloid Leukaemia

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Summary

Approximately 50% of children with acute myeloid leukaemia (AML) relapse, despite aggressive chemotherapy. The bone marrow stromal environment protects leukaemia cells from chemotherapy (i.e., stroma-induced chemoresistance), eventually leading to recurrence. Our goal is to delineate the mechanisms underlying stroma-mediated chemoresistance in AML. We used two human bone marrow stromal cell lines, HS-5 and HS-27A, which are equally effective in protecting AML cells from chemotherapy-induced apoptosis in AML-stromal co-cultures. We found that *CYR61* was highly expressed by stromal cells, and was upregulated in AML cells by both stromal cell lines. CYR61 is a secreted matricellular protein and is associated with cell-intrinsic chemoresistance in other malignancies. Here, we show that blocking stromal CYR61 activity, by neutralization or RNAi, increased mitoxantrone-induced apoptosis in AML cells in AML-stromal co-cultures, providing functional evidence for its role in stroma-mediated chemoresistance. Further, we found that spleen tyrosine kinase (SYK) mediates CYR61 signalling. Exposure to stroma increased SYK expression and activation in AML cells, and this increase required CYR61. SYK inhibition reduced stroma-dependent mitoxantrone resistance in the presence of CYR61, but not in its absence. Therefore, SYK is downstream of CYR61 and contributes to CYR61-mediated mitoxantrone resistance. The CYR61-SYK pathway represents a potential target for reducing stroma-induced chemoresistance.

Keywords

marrow stroma; adhesion; drug resistance; signalling; myeloid leukaemia

Acute myeloid leukaemia (AML) is a devastating malignancy of the bone marrow in which haematopoietic precursors undergo uncontrolled proliferation and arrest in an early stage of

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Author contributions

Drs. XL and LP performed the experiments and acquired the data; Drs. MSR, XL, YY, LP and T-K Man contributed to the research design, data analysis and the manuscript writing.

Conflict of interest

The authors have no competing interests.

development as myeloid blasts. Childhood AML has a high relapse rate and subsequent low survival rate, despite very toxic chemotherapy (Rubnitz *et al*, 2010; Gamis *et al*, 2014). Therefore new, rational therapies to circumvent chemotherapy resistance are desperately needed.

De novo chemotherapy resistance is present at the outset, in contrast to acquired chemotherapy resistance, which develops as a result of selective pressure during treatment (Meads *et al*, 2009). *De novo* resistance includes environment-mediated drug resistance (EMDR) (Meads *et al*, 2008, 2009). The tumour microenvironment is composed of stromal cells, extracellular matrix (ECM) and soluble factors, all of which can contribute to drug resistance (Matsunaga *et al*, 2003; Li & Dalton, 2006; Meads *et al*, 2008; Shain *et al*, 2009). Human and mouse bone marrow stromal cells have been shown to protect cells of various haematological malignancies against chemotherapy-induced apoptosis, thereby allowing disease recurrence (Mudry *et al*, 2000; Konopleva *et al*, 2002; Nefedova *et al*, 2003; Sison & Brown, 2011). In one salient report, over 700 anti-cancer drugs were less active against cancer cells when co-cultured with stromal cells (McMillin *et al*, 2010).

Stroma-mediated drug resistance includes soluble factor-dependent drug resistance and contact-dependent drug resistance (Meads *et al*, 2008, 2009). Soluble factors refer to cytokines, chemokines and growth factors secreted by stromal cells, which bind to their receptors and activate signalling pathways in malignant cells (Li & Dalton, 2006). Contact-dependent interactions exist between tumour and stromal cells, and between tumour cells and ECM. The ECM is composed of large molecular weight proteins like collagens and fibronectin, as well as smaller proteins, including the CCN family of matricellular ligands. The CCN family was named after cysteine-rich 61 (CYR61/CCN1), connective tissue growth factor (CTGF/CCN2) and nephroblastoma overexpressed (NOV/CCN3) (Perbal, 2004). As a secreted protein, CYR61 binds to integrins (Perbal, 2004), and has been linked to chemotherapy resistance in solid tumours (Lin *et al*, 2004; Menendez *et al*, 2005; Lai *et al*, 2011). However, the contribution of CYR61 to stroma-induced chemoresistance in leukaemia has not yet been described.

In the present study, we sought to determine the underlying mechanisms by which the bone marrow stroma protects AML cells from chemotherapy. Two human bone marrow stromal cell lines, HS-5 and HS-27A, were used. Both provide physical contact with malignant cells, but HS-5 cells secrete many more soluble factors than HS-27A cells (Roecklein & Torok-Storb, 1995). Using an *in vitro* AML-stroma co-culture model, we identified CYR61 as a mediator of resistance to mitoxantrone. Further, we demonstrated that CYR61-dependent resistance occurs via activation of SYK. SYK is a non-receptor tyrosine kinase widely expressed in haematopoietic cells. SYK is activated downstream of integrin ligation, and inhibition of SYK has been shown to induce apoptosis in AML models (Hahn *et al*, 2009; Miller *et al*, 2013). This is the first report of a CYR61-SYK pathway mediating stroma-induced chemotherapy resistance in AML.

Materials and methods

Reagents

Etoposide, mitoxantrone and cytarabine were obtained from the Texas Children's Hospital pharmacy. Selective SYK inhibitor R406 was purchased from Selleck (Houston, TX, USA). CYR61 neutralizing antibody was purchased from Novus Biologicals (Littleton, CO, USA). Normal rabbit IgG was from R&D Systems (Minneapolis, MN, USA). Hoechst 33342 was from Sigma-Aldrich (St. Louis, MO, USA).

Cell lines

NB-4 and THP-1 human AML cells were gifts of Drs. Shuo Dong and Terzah Horton (Baylor College of Medicine, Houston, TX, USA), respectively. Kasumi-1 human AML cells, HS-5, HS-27A human stromal cells and HEK293T cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). HS-5 and HS-27A cells were stably transduced with pCDH.CMV.mOrange (gift of Dr. Stephen Gottschalk, Baylor College of Medicine), to allow distinction from AML cells by flow cytometry. For all experiments except where indicated, HS-5-mOrange and HS-27A-mOrange cells are denoted as HS-5 and HS-27A cells. HS-5 and HEK293T cells were maintained in Dulbecco's modified Eagle's medium (Gibco/Life Technologies, Grand Island, NY, USA) with 10% foetal bovine serum (FBS, Invitrogen/Life Technologies Carlsbad, CA, USA), 2 mM L-Glutamine (Invitrogen), 100 units/ml penicillin and 100 µg/ml streptomycin (Pen/Strep; Invitrogen). All other cell lines were maintained in RPMI-1640 medium (ATCC) with 10% FBS and other additives as above. All cells were grown in a 90% humidified, 37°C incubator with 5% CO₂.

Primary AML samples

De-identified primary paediatric AML samples were obtained from the Research and Tissue Support Services (RTSS) Core of Texas Children's Cancer and Hematology Centers at Texas Children's Hospital and from the Children's Oncology Group (COG) AML Reference Laboratory (Fred Hutchinson Cancer Research Center, Seattle, WA, USA). All samples were enriched for mononuclear cells by density centrifugation, and cryopreserved. Samples were carefully thawed into warm Iscove's minimal essential medium with 20% FBS, and rested for 2 h before counting viable cells. Only samples with <40% spontaneous apoptosis after overnight culture were analysed. Written informed consent was obtained in accordance with the Declaration of Helsinki, for bone marrow and peripheral blood cells to be stored for research. These studies were approved by the Institutional Review Board of Baylor College of Medicine. Basic clinical data associated with the samples used in this study are provided in Table SI.

Co-culture and Transwell co-culture experiments

For co-culture experiments, human AML cell lines (NB-4, THP-1) or primary AML patient samples were seeded at 1×10^5 cells/ml and co-cultured with HS-5 or HS-27A cells pre-seeded the day before (ratio of AML:stromal cells 1:1), for 1 h (primary patient samples) or 24 h (cell lines). Cells were then treated with etoposide, mitoxantrone or cytarabine at

increasing doses for 24 h before performing Annexin V apoptosis assays. As a control, AML cells were cultured alone and treated with chemotherapy drugs in parallel. For the Transwell® co-culture experiments, AML cell lines were plated inside the Transwell® microporous inserts (0.4 µm pore size, polyester or polycarbonate) while stromal cells were seeded on the Transwell® plate surface (Corning Incorporated, Corning, NY, USA), underneath the inserts (ratio of AML:stromal cells 1:1), thus eliminating the physical contact between AML and stromal cells but allowing soluble factors to cross. The regimen of co-culture and chemotherapy treatment was the same as standard co-culture. Polycarbonate membranes were used for mitoxantrone treatment after preliminary experiments indicated that mitoxantrone adsorbs to the polyester membrane.

Annexin V apoptosis assay

For the apoptosis assay, cells were labelled using fluorescein isothiocyanate (FITC) Annexin V (BD Biosciences, San Jose, CA, USA). The mOrange-positive stromal cells were excluded, and the percentage of apoptotic AML cells (FITC-positive and mOrange-negative) was determined from 10 000 mOrange-negative events acquired for each sample by LSRII (BD Biosciences). Data were acquired and analysed with Diva (BD Biosciences). The percentage of spontaneous apoptosis was subtracted from the drug-treated samples to yield the percentage of apoptosis attributed to drug treatment, as described (Redell *et al*, 2011).

Multiplex cytokine assay

Medium was conditioned by HS-5 and HS-27A parental and mOrange stromal cells for 48 h at 1×10^5 /ml, and fresh RPMI medium with 10% FBS was used to define the background. Forty-two soluble factors in freshly harvested stroma-conditioned medium were quantified with the MIL-LIPLEX® MAP Human Cytokine/Chemokine Magnetic Bead Panel (Millipore, Billerica, MA, USA), using the Luminex 200™ (Luminex Corporation, Austin, TX, USA).

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

AML cells were cultured alone, or co-cultured with HS-5 or HS-27A cells for 24 h (primary paediatric samples) or 48 h (cell lines) before mOrange-negative AML cells were sorted by fluorescence-activated cell sorting (FACS) (MoFlo, Beckman Coulter, Indianapolis, IN, USA) at Flow Cytometry Core at Texas Children's Cancer and Hematology Centers. Total RNA from AML cell lines was extracted, and reverse transcribed into cDNA as described (Redell *et al*, 2011). Messenger RNA from primary samples was isolated and cDNA synthesized using µMACS mRNA isolation kit and cDNA synthesis module (Miltenyi Biotec, San Diego, CA, USA). The cDNA was used as the template in qRT-PCR (ABI Prism 7900HT; Applied Biosystems/Life Technologies). Parallel reactions were performed for *GAPDH*, which served as endogenous control for normalization. The fold change in mRNA levels for the co-culture conditions, relative to the culture alone conditions, was calculated by the $\Delta\Delta C_t$ method (Ono *et al*, 2004).

Oligonucleotide microarray assay and data analysis

Human AML cells were cultured for 48 h under 3 conditions: alone, co-culture with HS-5 cells or co-culture with HS-27A cells. Cells were then sorted by FACS (MoFlo, Flow Cytometry Core at Texas Children's Cancer and Hematology Centers) to isolate the mOrange-negative population. Three biological replicates were prepared for each culture condition, for two AML cell lines (NB-4 and THP-1), yielding a total of 18 samples. Total RNA was isolated and analysed for acceptable quantity and purity. Oligonucleotide microarray gene expression profiling was performed with the Affymetrix U133Plus 2 platform (Affymetrix, Santa Clara, CA, USA) by the Genomic and RNA Profiling Core at Baylor College of Medicine. The raw intensity data were log transformed, normalized and summarized by the Robust Multichip Average (RMA) algorithm (Irizarry *et al*, 2003). The invariant genes were filtered if <20% of expression value of a gene has at least a 1.5-fold change from the gene's median value or >50% missing intensity values. Then, the normalized intensities of the resulting genes were analysed by Significance Analysis of Microarray (SAM) to perform class comparisons of alone v. co-culture with HS-5 or HS-27A cells (Tusher *et al*, 2001). Genes, denoted by specific probesets, with a proportion of false discovery (PFD) 0.1 at 90th percentile after 1000 permutations were considered to be significant. The data were analysed by BRB ArrayTools, developed by Dr. Richard Simon and the BRB-Array Tools Development Team (Simon *et al*, 2007). The functional analysis of the significantly altered genes was performed using Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA, USA).

Immunocytochemistry

As described above, AML cells were co-cultured with stromal cells or cultured alone, and sorted for the mOrange-negative population. AML cells were then cytospun onto glass slides, fixed, and permeabilized as described (Perlaky *et al*, 1992). Slides were stained with rabbit-anti-human CYR61 primary antibody (Abcam, Cambridge, MA, USA) and donkey-anti-rabbit Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). The cells were co-stained for myeloperoxidase (MPO) using mouse-anti-human MPO primary antibody (Abcam) and chicken-anti-mouse Alexa Fluor® 488 secondary antibody (Invitrogen/Life Technologies). In parallel, the primary antibodies were omitted for some slides, which showed no non-specific binding by secondary antibodies themselves. MPO is a diagnostic marker for AML, and was used to confirm that CYR61-expressing cells were AML cells. Cell nuclei were counterstained with Hoechst 33342. The images were captured by Nikon Eclipse TE300 Fluorescent Microscope (Nikon, Tokyo, Japan) and analysed with Roper Scientific Image software (Media Cybernetics, Inc., Rockville, USA) at the RTSS Core, or with Nikon A1R Confocal Microscope (Nikon) and analysed with NIS Elements AR software (Nikon) at the Integrated Microscopy Core at Baylor College of Medicine.

CYR61 neutralization assays

THP-1 cells, as well as stromal cells, were pre-incubated with 20 µg/ml rabbit-anti-human CYR61 neutralizing antibody or normal rabbit IgG for 1 h at 37°C before either co-culture with stromal cells or continuing culture alone for 24 h. Then cells were treated with

etoposide (1.5 μ M) or mitoxantrone (50 nM) for 24 h, with addition of CYR61 neutralizing antibody or normal rabbit IgG again, followed by Annexin V apoptosis assays.

CYR61 knockdown

Packaging plasmids (VSV-G, Tat1b, Hgpm2, Rev1b) and lentivirus expression pGIPZ plasmids with short hairpin RNA (shRNA) constructs targeting human *CYR61*, or non-silencing control (purchased from the Cell-Based Assay Screening Service, C-BASS, core facility at Baylor College of Medicine), were added to HEK293T cells along with Lipofectamine 2000 (Invitrogen/Life Technologies). Stromal cells were transduced with lentiviral supernatant from HEK293T producer cells 48 h and 72 h after plasmid packaging with addition of 4 μ g/ml polybrene (Sigma-Aldrich). Transduced stromal cells were confirmed with >97% transduction efficiency with turboGFP and then selected with 0.5 μ g/ml puromycin (Sigma-Aldrich) for 5 days.

Western blotting

AML and stromal cells were treated with Brefeldin A (BD Biosciences, 5 μ l/ml culture medium) and monensin (BD Biosciences, 2.5 μ l/ml culture medium) for 5 h in order to block secretion of CYR61. Preparation of whole cell protein lysate and Western blotting were performed as described (Redell *et al*, 2011). CYR61 was detected with rabbit-anti-human CYR61 neutralizing antibody (1:200 dilution) and Alexa Fluor[®] 680 goat-anti-rabbit antibody (1:5000 dilution, Invitrogen/Life Technologies) on an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). β -actin (ACTB, Sigma-Aldrich) served as loading control.

Intracellular flow cytometry

AML cells were cultured alone, or co-cultured with control/CYR61-KD HS-27A stromal cells for 24 h (primary patient samples) or 48 h (cell lines), before they were harvested, fixed, permeabilized and intracellularly stained as described (Redell *et al*, 2011). Cells were stained with Alexa Fluor[®] 488 anti-pY348-SYK (BD Biosciences), Alexa Fluor[®] 647 anti-pY352-SYK (BD Biosciences), phycoerythrin (PE) anti-pY525/526-SYK (Cell Signaling Technology, Danvers, MA, USA), FITC anti-SYK (BD Biosciences), or isotype control and analysed by LSRII. The mOrange-positive stromal cells were excluded, and 10 000 mOrange-negative events were acquired for AML cells. Similarly, Kasumi-1 AML cells were serum starved for 1 h, then stimulated with 100 ng/ml granulocyte colony-stimulating factor (G-CSF), HS-5- or HS-27A- conditioned medium for 15 min, before they were harvested, fixed, permeabilized, and intracellularly stained with PE anti-pY705-STAT3 (BD Biosciences) or isotype control as described above. 10 000 events were acquired and analysed for Kasumi-1 cells by LSRII. Data were further analysed with FCS Express 4 (De Novo Software, Glendale, CA, USA).

Inhibition of p-SYK by R406

NB-4, THP-1 cells or primary AML patient samples were pre-incubated with 3 μ M R406 or dimethyl sulfoxide (DMSO) for 30 min at 37°C before either co-culture with control/ CYR61-KD HS-27A cells, or continuing culture alone, for 1 h (primary patient samples) or

24 h (cell lines). Then all cells were treated with 300 nM mitoxantrone for 24 h before Annexin V apoptosis assays.

Statistical analysis

Values are means $\pm$ standard error of the mean (SEM). Dose-response apoptosis curves were generated by 4-parameter logistic equation using GRAPHPAD PRISM 5.02 software (GraphPad Software Inc., La Jolla, CA, USA). Two-way analysis of variance was used to compare co-culture v. alone or CYR61 neutralization v. rabbit IgG under different conditions, followed by Bonferroni's least significant difference post hoc test using GraphPad Prism. Unpaired, two-tailed, t-test was used to compare apoptosis rates for AML cells in co-culture with non-silencing control (NS Ctrl) stroma v. CYR61-KD stromal cells, treated with different chemotherapies. $P < 0.05$ was considered significant.

Results

HS-5 and HS-27A stromal cells have distinct profiles of secreted soluble factors

In order to be able to distinguish stromal cells from leukaemia cells after co-culture, we stably transduced HS-5 and HS-27A cells with mOrange. To compare HS-5 to HS-27A cells, and to confirm the similarity of mOrange-transduced to parental stromal cells, we first examined the profiles of soluble factors in stroma-conditioned medium by performing a multiplex cytokine assay for 42 secreted factors. HS-5 cells secreted high levels of over 20 factors, including G-CSF and interleukin-6 (IL6), while HS-27A cells secreted only a few, e.g., vascular endothelial growth factor (VEGFA 165), monocyte chemoattractant protein-1 (MCP1, or chemokine C-C motif ligand 2, CCL2) and IL8 (Fig 1A–D). This assay also confirmed that the soluble factor secretion profiles were similar between parental and mOrange stromal cells.

Given that G-CSF and IL6 are known activators of STAT3 (Redell *et al*, 2011, 2013), and that both are highly abundant in HS-5-conditioned medium but not in HS-27A-conditioned medium (Fig 1C), we next compared the stroma-conditioned media in the ability to activate STAT3 in Kasumi-1 AML cells. We found that HS-5-conditioned medium, but not HS-27A-conditioned medium, induced STAT3 phosphorylation (Y705) in Kasumi-1 cells, similar to treatment with G-CSF alone (Fig S1A). Again, conditioned media from the mOrange-transduced and parental stromal cells were equally effective in activating STAT3 in Kasumi-1 cells (Fig S1B).

Both stromal cell lines protected AML cell lines and primary AML patient samples from chemotherapy-induced apoptosis

After validation of the mOrange-transduced stromal cells, we established an *in vitro* model of the bone marrow environment by co-culturing AML cells with mOrange-transduced stromal cells. We tested the ability of HS-5 and HS-27A cells to protect AML cells from apoptosis induced by exposure to etoposide, mitoxantrone or cytarabine, three chemotherapy drugs used to treat AML patients. Both stromal cell lines significantly protected NB-4 cells (Fig 2A1–3) and THP-1 cells (Fig 2B1–3) from apoptosis induced by all three chemotherapy drugs in a dose-dependent manner. Interestingly, the two stromal cell lines conferred

equivalent levels of resistance to etoposide and mitoxantrone. For cytarabine, HS-5 co-culture was more protective than HS-27A co-culture at intermediate drug doses (Fig 2A2, B2; Annexin V positive rate of NB-4 treated with 3 μ M cytarabine was $5.1 \pm 0.6\%$ for HS-5 co-culture vs $13.2 \pm 2.0\%$ for HS-27A co-culture, $P < 0.05$).

In addition to AML cell lines, we tested the ability of both stromal cell lines to protect primary paediatric AML patient samples against these chemotherapy agents. We found that primary AML cells were also significantly and similarly protected from chemotherapy-induced apoptosis by co-culture with both stromal cell lines (Fig 2C, D). Both stromal cells also protected primary patient samples against spontaneous apoptosis at baseline (data not shown). These results support the assertion that the stroma-mediated chemotherapy resistance demonstrated in AML cell lines is similar to the resistance that occurs in patients.

Different stroma-dependent mechanisms contribute to resistance for different chemotherapies

To investigate the underlying mechanisms of stroma-induced chemotherapy resistance for AML cells, we performed Transwell co-culture experiments. The Transwell inserts provide a microporous membrane which allows soluble factors, but not cells, to cross, thus preventing physical contact between the AML cells on top of the insert and the stromal cells on the well surface, below the membrane. In contrast to co-culture with direct contact (Fig 2A1, B1), Transwell co-culture significantly abrogated the protection conferred by both stromal cell lines against etoposide, for both NB-4 cells and THP-1 cells (Fig 3A1, B1; Annexin V positive rate of NB-4 treated with 3 μ M etoposide was $24.1 \pm 3.7\%$ for HS-5 Transwell co-culture vs $29.8 \pm 5.3\%$ for NB-4 cells alone, not significant). Further, incubation of NB-4 and THP-1 cells with stroma-conditioned medium before and during etoposide treatment had very little protective effect on apoptosis (Fig S2A, B). These findings indicated that the mechanism of stroma-induced protection from etoposide in AML cells mainly requires direct cell-cell contact.

For cytarabine treatment, the Transwell membrane reduced the protection from HS-5 cells, suggesting some contribution from contact-dependent pathways to HS-5-induced cytarabine resistance. However, both cell lines still provided significant protection, suggesting both stromal cell lines also secrete soluble factors that promote resistance to cytarabine (Fig 3A2, B2). For example, the Annexin V positive rate of NB-4 cells treated with 3 μ M cytarabine was $9.0 \pm 1.1\%$ for HS-5 Transwell co-culture and $11.6 \pm 2.0\%$ for HS-27A Transwell co-culture, vs. $20.0 \pm 4.0\%$ for NB-4 cells alone ($P < 0.05$). A protective effect of soluble factors was further supported by the finding that both stroma-conditioned media significantly protected NB-4 and THP-1 cells from cytarabine (Fig S2C, D).

Stroma-induced resistance to mitoxantrone also was partially reduced by the Transwell inserts, with more reduction of HS-27A- than HS-5-induced resistance (Fig 3A3, B3). The Annexin V positive rate of NB-4 cells treated with 100 nM mitoxantrone was $30.7 \pm 3.4\%$ for HS-27A Transwell co-culture vs $22.2 \pm 2.3\%$ for HS-5 Transwell co-culture ($P < 0.05$). A substantial role for HS-5-derived soluble factors was confirmed by our finding that HS-5-conditioned medium significantly reduced mitoxantrone-induced apoptosis (Fig S2E, F; Annexin V positive rate of NB-4 cells treated with 300 nM mitoxantrone was $35.0 \pm 1.1\%$

for HS-5-conditioned medium vs $50.9 \pm 1.0\%$ for control medium, $P < 0.001$). In contrast, HS-27A-conditioned medium provided minimal protection against mitoxantrone. Taken together, our results indicated that resistance to mitoxantrone, and to a lesser extent cytarabine, can be conferred by soluble factors secreted by stromal cells, while direct contact with stromal cells confers resistance to all three chemotherapy agents.

Stroma-induced genes and proteins in AML cells

To search for candidate genes responsible for stroma-dependent chemoresistance in AML cells, we compared the gene expression profiles of NB-4 and THP-1 AML cell lines under 3 conditions: cultured alone, co-cultured with HS-5 cells, or co-cultured with HS-27A cells. After 48 h in culture, cells were flow-sorted to isolate the mOrange-negative population. RNA was extracted for gene expression profiling using the Affymetrix U133Plus2 platform. Forty genes were found to be significantly up-regulated in both NB-4 and THP-1 AML cells after co-culture with HS-5 cells (Table SII), while over 1000 genes were altered, either up- or down-regulated, in both AML cell lines after co-culture with HS-27A cells (Tables SIII, SIV). Additionally, using SAM analysis, we identified genes that were more robustly induced in the AML cells by one stromal cell line compared to the other, and therefore distinguish between the two stromal cell lines (PFD < 0.1). The 29 differentially expressed probesets, representing 24 genes, are listed in Table SV. For example, *CD52*, *RPS6* (ribosomal protein S6), and *FGR* (FGR proto-oncogene, Src family tyrosine kinase) were all more robustly upregulated in AML cells by HS-27A cells than by HS-5 cells. *GREM1* [gremlin 1, cysteine knot superfamily, homolog (*Xenopus laevis*)], *ABI3BP* [ABI family, member 3 (NESH) binding protein], and *SERPINB2* [serpin peptidase inhibitor, clade B (ovalbumin), member 2] were more upregulated by HS-5 than by HS-27A cells.

To further evaluate the functions of those differentially expressed stroma-induced genes with regard to drug resistance, functional ontology analysis of the significantly differentially expressed genes was performed using Ingenuity Pathway Analysis. In the Diseases and Disorders category, Cancer was the most significantly enriched. Over 30 genes induced by HS-5 cells and over 100 genes induced by HS-27A cells were related to cancer. In the Molecular and Cellular Function category, the stroma-induced gene lists were significantly enriched in functions including cellular growth, cell-cell signalling and cell death (Table SVI).

Of particular interest were 19 transcripts that were commonly increased in both AML cell lines after co-culture with both stromal cell lines (Fig 4A, Table SVII). Given that both stromal cells provided similar degrees of protection for AML cells against chemotherapy, those 19 genes represent likely candidates contributing to chemoresistance. In addition, several genes, including *CYR61*, *CAV1* (Caveolin 1, caveolae protein, 22 kDa), *CCL2*, *CTGF* and *AXL* (AXL receptor tyrosine kinase) are associated with chemotherapy resistance in other malignancies (Ho *et al*, 2008; Hong *et al*, 2008; Qian *et al*, 2010; Yin *et al*, 2010; Lai *et al*, 2011). To verify stroma-upregulated transcripts, we cultured AML cells alone or co-cultured with either stromal cell line, flow sorted to isolate the mOrange-negative population, and extracted RNA for qRT-PCR. We confirmed the stroma-induced upregulation of *CYR61* (Fig 4B), as well as *CAV1*, *TM4SF1* (Transmembrane-4-L-six-

family-1), *CTGF*, *CCL2* and *AXL* (data not shown), in both AML cell lines and in primary samples.

Next, we examined the protein expression for selected genes by immunocytochemistry. AML cells were co-cultured with stromal cells or cultured alone, sorted for the mOrange-negative population, cytopun onto glass slides, fixed, permeabilized and stained for candidate stroma-upregulated proteins. The cells were co-stained for MPO, a clinically used marker for AML, in order to confirm that the imaged cells were AML cells and not contaminating stromal cells. CYR61, TM4SF1 and CCL2 proteins were detected in AML cells cultured alone (data not shown). Of these, only CYR61 protein was clearly up-regulated in THP-1 cells and primary paediatric AML samples (Fig 4C) after co-culture with HS-5 stromal cells. Similar CYR61 upregulation was detected in THP-1 cells and primary AML cells co-cultured with HS-27A cells and NB-4 cells co-cultured with either stromal cells (data not shown). These results suggested that stromal cells may confer resistance to chemotherapy partly through the upregulation of CYR61.

Reducing CYR61 activity partially reversed the stroma-induced resistance to mitoxantrone in AML cells

As an ECM-associated signalling molecule, CYR61 promotes cell adhesion, the mitogenic effects of growth factors (Kireeva *et al*, 1996; Babic *et al*, 1998), and taxol resistance in breast cancer (Menendez *et al*, 2005), so we hypothesized that CYR61 contributed to stroma-induced chemotherapy resistance in AML. We used anti-CYR61 neutralizing antibody to block CYR61 activity in THP-1 cells cultured alone or co-cultured with stromal cells, and treated with chemotherapy. Neutralization of CYR61 did not alleviate stroma-induced resistance to etoposide in THP-1 cells (Fig 5A). However, blocking CYR61 activity significantly reduced the protection provided by HS-27A cells to mitoxantrone, with a similar trend toward increased apoptosis in THP-1 cells co-cultured with HS-5 cells or cultured alone (Fig 5B; Annexin V positive rate of THP-1 cells co-cultured with HS-27A cells and treated with 50 nM mitoxantrone was $30.9 \pm 4.0\%$ for CYR61 neutralizing antibody vs $12.6 \pm 3.0\%$ for rabbit IgG control, $P < 0.05$).

Next, we wanted to confirm the pharmacological neutralization results by RNAi knockdown. Although CYR61 expression was increased in co-cultured AML cells, we found that stromal cells express much higher levels of CYR61 at baseline, and therefore stromal cells are likely to be the major source of CYR61 in the bone marrow (Fig S3). Therefore, we transduced both HS-5 and HS-27A stromal cells with lentiviral supernatant containing shRNA constructs targeting *CYR61*, then confirmed the reduction in expression by qRT-PCR (Fig 6A) and Western blotting (Fig 6B). Among the 4 different shRNA constructs targeting *CYR61*, stromal cells transduced with constructs 2 (C2) and 4 (C4) achieved the highest knockdown efficiency, compared to stroma transduced with non-silencing control (NS Ctrl). In regards to their function, the mitoxantrone resistance conferred by C4-transduced stromal cells was significantly reduced, compared to NS Ctrl-transduced stromal cells (Fig 6C, D). The Annexin V positive rate of NB-4 cells co-cultured with HS-27A cells and treated with 100 nM mitoxantrone was $12.2 \pm 0.7\%$ for co-culture with C4-transduced HS-27A vs. $7.2 \pm 0.7\%$ for co-culture with NS Ctrl-transduced HS-27A ($P < 0.05$). Similarly, the Annexin V

positive rate of THP-1 cells was $10.8 \pm 0.8\%$ for co-culture with C4-transduced HS-27A v. $6.8 \pm 1.1\%$ for co-culture with NS Ctrl-transduced HS-27A ($P < 0.05$). However, the resistance to etoposide and cytarabine was not changed by CYR61 knockdown in HS-27A stromal cells (Fig 6E–H). The results of these CYR61 knockdown experiments are consistent with the CYR61 neutralization results, and support a role for CYR61 in mediating stroma-induced resistance to mitoxantrone in AML cells.

CYR61 increases SYK expression and SYK activation

According to the microarray mRNA gene profiling, *SYK* was among the genes in AML cells that were upregulated by co-culture with HS-27A cells (Table SIII). Given that CYR61 is reported to bind to integrin $\beta 3$ (Menendez *et al*, 2005; Su *et al*, 2010; Long *et al*, 2013), and integrin $\beta 3$ signals through SYK in AML cells (Miller *et al*, 2013), we hypothesized that SYK may be downstream of CYR61 in mediating HS-27A-induced resistance to mitoxantrone. We first determined total SYK (t-SYK) expression in AML cells cultured alone or co-cultured with NS Ctrl or CYR61-KD HS-27A cells by flow cytometry. T-SYK was upregulated in NB-4 and THP-1 cells co-cultured with NS Ctrl HS-27A, but not in AML cells co-cultured with CYR61-KD HS-27A (Fig 7A, B). Next, we measured SYK activation by phospho-SYK (p-SYK) in AML cells, in the same culture conditions, by flow cytometry. As the phosphorylation of tyrosine 348 (Y348) and Y352 of SYK is important for its conformational activation (Gradler *et al*, 2013) and pY525/526 also contributes to its tyrosine kinase activity (Zhang *et al*, 2000), we investigated pY348, pY352 and pY525/526 SYK. Levels of pY348 were significantly increased in NB-4 and THP-1 cells co-cultured with NS Ctrl HS-27A, but not in cells co-cultured with CYR61-KD HS-27A (Fig 7C, D). The mean fluorescence intensity (MFI) of pY348 was 19.0 ± 4.3 for NB-4 co-cultured with NS Ctrl HS-27A vs. 10.1 ± 1.6 for NB-4 co-cultured with CYR61-KD HS-27A ($P < 0.05$), and 7.5 ± 1.0 for NB-4 alone, ($P < 0.05$). Similarly, pY348 was significantly increased in primary AML samples after co-culture with NS Ctrl HS-27A cells, but not after co-culture with CYR61-KD cells (Fig 7E). In contrast, pY352 and pY525/526 did not change in NB-4 and THP-1 cells co-cultured with NS Ctrl HS-27A (Fig S4A–D). These results suggested that the increase in stroma-induced SYK activation was specific for pY348 and was mediated by the ECM ligand, CYR61.

CYR61-induced SYK activation confers resistance to mitoxantrone

To determine the role of SYK in CYR61-mediated mitoxantrone resistance, we used the small molecule SYK inhibitor, R406 (Brasemann *et al*, 2006). To determine the optimal dose of R406 for inhibition of SYK activation (pY348), we pre-treated AML cells with different doses of R406, prior to co-culture with NS Ctrl HS-27A and measured p-SYK (Y348) in AML cells by flow cytometry. At 3 μ M, R406 achieved significant inhibition of SYK activation (pY348) in AML cells co-cultured with NS Ctrl HS-27A (Fig 8A, B), but not in AML cells cultured alone or co-cultured with CYR61-KD HS-27A (data not shown), further supporting the notion that SYK activation via pY348 is mediated by stromal CYR61.

Next, we pre-treated NB-4, THP-1 cells and primary AML patient samples with 3 μ M R406, and then either cultured alone or co-cultured with NS Ctrl HS-27A or CYR61-KD HS-27A and treated with mitoxantrone for 24 h. R406 treatment of AML cells cultured alone did not

increase apoptosis, indicating that this drug is not directly toxic to AML cells (Fig 8C, D). In contrast, R406 treatment of AML cells co-cultured with NS Ctrl HS-27A cells did result in increased mitoxantrone-induced apoptosis, suggesting that p-SYK (Y348) contributes to this stroma-mediated resistance pathway (Fig 8C, D; Annexin V positive rate of NB-4 cells co-cultured with NS Ctrl HS-27A cells and treated with 300 nM mitoxantrone was $29.6 \pm 3.1\%$ for R406 vs. $20.4 \pm 1.8\%$ for DMSO, $P < 0.05$). Importantly, R406 did not further reduce mitoxantrone resistance beyond that already achieved by CYR61 knockdown (Fig 8C, D). Likewise, in primary paediatric AML samples, inhibition of SYK activity dramatically reversed mitoxantrone resistance conferred by CYR61-expressing stromal cells (Fig 8E). The Annexin V positive rate for primary AML cells co-cultured with NS Ctrl HS-27A cells and treated with 300 nM mitoxantrone was $71.4 \pm 9.3\%$ for R406 vs. $14.8 \pm 18.1\%$ for DMSO ($P < 0.05$). The difference in apoptosis achieved by SYK inhibition in primary AML cells co-cultured with CYR61-KD stromal cells was not significant (Fig 8E). These results place SYK (Y348) downstream of CYR61 in mediating HS-27A-induced mitoxantrone resistance in AML cell lines as well as primary patient samples.

Discussion

In the current study, we used an *in vitro* model of the bone marrow microenvironment to investigate the mechanisms of stroma-mediated chemotherapy resistance in AML, and to identify targets for overcoming this resistance. We compared the effects of co-culture with two distinct stromal cell lines, HS-5 and HS-27A. Both provided similar degrees of protection for AML cells against three chemotherapy drugs used clinically to treat patients with AML. However, chemoresistance is a diverse and complex process, as evidenced by our findings that the two cell lines confer resistance by different mechanisms, and that resistance to different chemotherapy agents can be achieved via different routes.

HS-5 and HS-27A stromal cell lines are both derived from the fibroblast lineage, but they differ in morphology, gene expression patterns (Graf *et al*, 2002), and secreted factors (Roecklein & Torok-Storb, 1995). Using a comprehensive multiplex bead assay, we further defined the differences in soluble factor levels between HS-5 and HS-27A cells. Although HS-5 cells secrete many more soluble factors at higher levels than HS-27A cells, both stromal cells are able to maintain colony-forming cells at similar levels (Roecklein & Torok-Storb, 1995), and our results indicate that both are equally effective at promoting chemoresistance. It is somewhat surprising that a small number of genes were found to be significantly differentially expressed in the HS-5 co-culture relative to the HS-27A co-culture. The two AML cell lines may respond differently to soluble factors, due to different levels of receptors or intracellular mediators, for example, resulting in fewer genes meeting our selection criteria. This is supported by the fact that NB-4 and THP-1 cells showed different magnitudes of upregulation of *SOCS3* induced by G-CSF treatment (Redell *et al*, 2011). Also, we observed a higher variability between the replicates of AML cells co-cultured with HS-5 cells relative to those co-cultured with HS-27A cells. This would also decrease the power of detecting significant genes in the HS-5 co-culture condition.

Our work showed that adhesion and soluble factors differentially contributed to stroma-induced resistance to different chemotherapies. For example, we demonstrated that

resistance to etoposide requires direct contact with stromal cells, which is supported by previous work in pre-B acute lymphoblastic leukaemia (Mudry *et al*, 2000). In contrast, resistance to cytarabine and mitoxantrone has been reported to involve contact-dependent interactions and soluble factor-induced pathways (Mudry *et al*, 2000; Konopleva *et al*, 2002; Nefedova *et al*, 2003; Zeng *et al*, 2006), and our results implicate both processes for both drugs, as well. Etoposide induces DNA strand breaks by inhibition of topoisomerase II. It is most effective against cells in the G2 and S phases of the cell cycle. Mitoxantrone is also a potent inhibitor of topoisomerase II, but it can also intercalate into DNA and RNA. Mitoxantrone affects dividing and nondividing cells, so its actions are not cell cycle phase-specific. Cytarabine inhibits DNA polymerase and kills cells in the S phase and blocks the progression of cells from the G1 phase to the S phase. As etoposide and mitoxantrone both are topoisomerase II inhibitors, we believe that the mechanisms of action of the drugs do not fully explain the differences in stroma-dependent protective mechanisms. As for mitoxantrone and cytarabine, although they share similar protective effects induced by stroma (direct contact or soluble factor-induced), their mechanisms of action are very distinct. Therefore, the mechanisms of stroma-induced resistance described here are likely to be independent of the functions of the drugs.

While the contributions of adhesion and of soluble factors are well-studied, the downstream signalling pathways involved have not been completely described. A very recent report from Jacamo *et al* (2014), identified increased nuclear factor (NF)- κ B signalling, induced in both stromal cells and leukaemia cells by VCAM1/VLA4 interaction, as a mediator of resistance to vincristine and to doxorubicin. Our study points to another pathway, in which the ECM ligand CYR61 increases SYK expression and activity in AML cells.

Our experiments led to the discovery of *CYR61* as a potential mediator of stroma-induced resistance to mitoxantrone. CYR61 promotes breast cancer and prostate carcinoma growth (Sampath *et al*, 2002; Franzen *et al*, 2009), enhances pancreatic and gastric carcinogenesis (Babic *et al*, 1998; Haque *et al*, 2011), and is linked to intrinsic chemotherapy resistance in breast cancer and cervical cancer (Rho *et al*, 2009; Lai *et al*, 2011). This is the first time CYR61 has been shown to mediate stroma-induced chemotherapy resistance and the first time that CYR61 has been studied in any haematological malignancy. As an ECM-associated signalling molecule, CYR61 is thought to promote cell attachment and potentiate the effects of growth factors on DNA synthesis (Kireeva *et al*, 1996; Babic *et al*, 1998). Therefore, in the context of AML, CYR61 may enhance the proliferation and survival of AML cells by augmenting their adhesion to stromal cells, or by increasing the secretion or the activity of pro-survival growth factors; both mechanisms lead to activation of downstream survival signalling pathways. We found that CYR61 was more abundant in HS-27A cells than in HS-5 cells, and that CYR61 inhibition, by neutralizing antibody and by knockdown, was more effective for reversing HS-27A-induced mitoxantrone resistance, compared to HS-5. Therefore, the CYR61 pathway may be more important for resistance conferred by HS-27A cells than HS-5 cells. It is interesting to note that CYR61 neutralization did not alter etoposide resistance or cytarabine resistance. As etoposide and mitoxantrone are both topoisomerase II inhibitors, this difference in susceptibility to CYR61 signalling implies that this pathway does not induce resistance via altered topoisomerase activity.

Previous studies have shown that NF- κ B (Lin *et al*, 2004), ERK1/2 (Menendez *et al*, 2005) and PI3K-AKT (Lai *et al*, 2011) are activated downstream of CYR61 and are associated with innate chemoresistance in breast cancer cells. In AML cells our gene expression profiling results led us to investigate SYK as a potential downstream mediator of CYR61-induced mitoxantrone resistance. SYK inhibition has been reported to inhibit cell proliferation, reduce cell viability, and increase cell apoptosis in AML (Hahn *et al*, 2009), and activated SYK promotes resistance to FLT3 inhibitors (Puissant *et al*, 2014). Thus, SYK may contribute to chemoresistance in AML. SYK includes two N-terminal Src Homology 2 (SH2) domains, one C-terminal kinase domain and two interdomain linkers. SYK is maintained in an autoinhibitory conformation in the basal condition. Upon appropriate stimulation, SYK undergoes autophosphorylation, or phosphorylation by Src family kinases, at Y348 and/or Y352 (Gradler *et al*, 2013), which disrupts the autoinhibitory conformation and causes its activation. Y348 and Y352 lie within the interdomain linker associated with the autoinhibitory conformation, and pY348 and pY352 are reported to be most important for SYK activation (Gradler *et al*, 2013). Y525/526 are located in the activation loop of the kinase domain, and pY525/526 may be less important for SYK activation than pY348 and pY352 (Gradler *et al*, 2013). Increased phosphorylation on Y348 therefore is expected to result in increased kinase activity. Our results confirmed a role for pY348 in SYK activation, and demonstrated for the first time a CYR61-SYK (pY348) pathway mediating stroma-dependent chemotherapy resistance. The confirmation of our results in patient-derived AML cells further validates the clinical relevance of this resistance mechanism. Understanding how the stromal microenvironment protects AML cells against chemotherapy, and how these processes may be overcome by inhibition of adhesion pathways, soluble factors, or their downstream targets, is likely to result in promising mechanism-oriented new therapies to reduce chemotherapy resistance and relapse, and thereby improve survival for patients with AML.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig 1.

HS-5 cells secreted many more factors than HS-27A cells. Both parental (pa) and mOrange (mO) stromal cells were cultured for 48 h, and 42 soluble factors were examined in the stroma-conditioned medium by multiplex cytokine assay. Detectable soluble factors are grouped by concentrations (A–D). $N = 4-5$.

Fig 2.

Bone marrow stromal cells conferred resistance to chemotherapy-induced apoptosis in acute myeloid leukaemia (AML) cell lines and primary paediatric patient samples. NB-4 and THP-1 AML cell lines and primary patient samples were kept in suspension, or co-cultured with HS-5 or HS-27A stromal cells, for 24 h (cell lines) or 1 h (patient samples), followed by exposure to chemotherapy drugs for 24 h before Annexin V apoptosis assay. Dose-response apoptosis curves of NB-4 (A1-3), THP-1 (B1-3), a representative patient sample (C1-3) and the averaged results from five patient samples (D1-3) are depicted. $N = 4-5$. BM, bone marrow mononuclear cells.

*, $P < 0.05$, alone v. HS-5 or HS-27A co-culture; #, $P < 0.05$, HS-5 v. HS-27A co-culture.

Fig 3.

Cell-cell contact and soluble factors differentially contributed to resistance to different chemotherapies in acute myeloid leukaemia (AML) cells. NB-4 and THP-1 AML cells were cultured alone, or co-cultured in Transwell inserts physically separated from stromal cells, and treated with chemotherapy before Annexin V apoptosis assay. Stroma-induced protection of AML cells from etoposide (A1, B1), cytarabine (A2, B2) and mitoxantrone (A3, B3) was shown. $N = 3-4$. *, $P < 0.05$, alone v. HS-5, or HS-27A co-culture; +, $P < 0.05$, alone v. HS-5 co-culture; #, $P < 0.05$, HS-5 v. HS-27A co-culture.

Fig 4.

A subset of stroma-induced genes was commonly upregulated by both stromal cell lines in acute myeloid leukaemia (AML) cells. NB-4 (N) and THP-1 (T) cells were cultured alone, or co-cultured with stromal cells for 48 h, then flow sorted to isolate the mOrange-negative population, from which total RNA was extracted for gene expression profiling. (A) Nineteen genes, including *CYR61*, were found upregulated in both AML cell lines by both stromal cell lines. The levels of those genes (mean-centred, denoted by gene-specific probesets) are shown. Red/green denotes relatively high/low gene expression, respectively. (B) *CYR61* mRNA level was determined by qRT-PCR in NB-4 ($N = 4$), THP-1 ($N = 4$), and two AML patient samples cultured alone, or co-cultured with stromal cells. *, $P < 0.05$, alone v. HS-5 or HS-27A co-culture. (C) *CYR61* protein expression was determined by immunocytochemistry in THP-1 cells and primary patient samples cultured alone or co-cultured with HS-5 cells. Representative images of *CYR61* (column 1), myeloperoxidase (MPO; AML marker, column 2) staining and merged images of *CYR61*, MPO, and Hoechst DNA staining for cell nuclei (column 3) are shown. The scale bars shown are 25 μ m.

Fig 5.

Neutralizing CYR61 reduced stroma-induced mitoxantrone resistance in THP-1 cells. THP-1 and stromal cells were pre-incubated with anti-CYR61 neutralizing antibody or normal rabbit IgG (both at 20 $\mu\text{g/ml}$) prior to placing in co-culture. After co-culture for 24 h, chemotherapy agent was added, along with anti-CYR61 or control IgG, and apoptosis was measured after another 24 h. The contributions of CYR61 activity to the stroma-induced protection from (A) 1.5 μM etoposide and (B) 50 nM mitoxantrone are shown. $N = 3$. *, $P < 0.05$, anti-CYR61 antibody v. normal rabbit IgG.

Fig 6.

CYR61 knockdown in stromal cells alleviated stroma-induced mitoxantrone resistance in NB-4 and THP-1 cells. HS-5 and HS-27A cells were transduced with shRNA constructs targeting *CYR61*. Knockdown efficiency was evaluated by (A) quantitative reverse transcription polymerase chain reaction and (B) Western blotting, respectively. Compared to non-silencing control (NS Ctrl), constructs 2 (C2) and C4 had the highest knockdown efficiency, and these two were used for further functional analyses. The contribution of stromal CYR61 to resistance to 100 nM mitoxantrone (C, D), 1.5 μ M etoposide (E, F) or 3 μ M cytarabine (G, H) was determined in AML cells co-cultured with C2- or C4-transduced stromal cells in comparison to those co-cultured with NS Ctrl stromal cells. $N = 4$. *, $P < 0.05$ as indicated.

Fig 7.

Stromal CYR61 upregulated total-SYK (t-SYK) and phospho-SYK (Y348) in acute myeloid leukaemia (AML) cells. T-SYK was determined by flow cytometry in NB-4 (A, $N = 3$) and THP-1 cells (B, $N = 3$), cultured alone or co-cultured with non-silencing control (NS Ctrl) HS-27A or CYR61-KD HS-27A cells for 48 h. P-SYK (Y348) was also determined in NB-4 (C, $N = 6$), THP-1 (D, $N = 4$) and paediatric AML patient samples (E, $N = 9$) in the similar culture conditions. The expression levels were quantified by mean fluorescence intensity (MFI). *, $P < 0.05$, **, $P < 0.01$, + NS Ctrl v. alone.

Fig 8.

Inhibition of p-SYK partially reversed HS-27A-induced mitoxantrone resistance. NB-4 (A, $N=3$) and THP-1 cells (B, $N=4$) were pre-incubated with SYK inhibitor R406 (30 nM, 300 nM, or 3 μ M) or dimethyl sulfoxide (DMSO) vehicle control for 30 min, before co-culture with NS Ctrl HS-27A cells for 48 h. Then p-SYK (Y348) was determined in acute myeloid leukaemia (AML) cells by flow cytometry and quantified by MFI. NB-4 (C, $N=4$), THP-1 cells (D, $N=3$) and primary AML patient samples (E, $N=5$) were pre-incubated with 3 μ M R406 or DMSO for 30 min, then were either cultured alone, or co-cultured with NS Ctrl HS-27A or CYR61-KD HS-27A cells. All cells were treated with 300 nM mitoxantrone for 24 h before the Annexin V apoptosis assay. The contribution of SYK activation (Y348) to stromal CYR61-induced resistance to mitoxantrone was depicted. *, $P < 0.05$ as indicated.