Distinct protein signatures of acute myeloid leukemia bone marrow-derived stromal cells are prognostic for patient survival

Steven M. Kornblau,1* Peter P. Ruvolo,1* Rui-Yu Wang,1 V. Lokesh Battula,1 Elizabeth J. Shpall,2 Vivian R. Ruvolo,2 Teresa McQueen,1 YiHua Qui,1 Zhihong Zeng,1 Sherry Pierce,1 Rodrigo Jacamo,1 Suk-Young Yoo,3 Phuong M. Le,2 Jeffrey Sun,1 Numsen Hail Jr,1 Marina Konopleva1 and Michael Andreeff1

1Section of Molecular Hematology and Therapy, Department of Leukemia; 2Department of Stem Cell Transplantation and 3Bioinformatics and Computational Biology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas, TX, USA

*SMK and PPR contributed equally to this work.

©2018 Ferrata Storti Foundation. This is an open-access paper. doi:10.3324/haematol.2017.172429

Received: May 9, 2017.
Accepted: February 1, 2018.
Pre-published: March 15, 2018.
Correspondence: skornblau@mdanderson.org or mandreef@mdanderson.org
Kornblau et al Supplemental Materials

Supplemental Methods

Isolation and culture of primary MSC from bone marrow

MSC were isolated from bone marrow (BM) of consented AML patients undergoing diagnostic BM aspiration and from healthy donors who were undergoing BM harvest for use in allogeneic BM transplantation. BM was subjected to centrifugation (700 g for 15 minutes at 4°C) over a Ficoll-Hypaque (Sigma-Aldrich) gradient to separate mononuclear cells. After centrifugation, the buffy coat layer was carefully extracted and suspended in αMEM (Cellgro, Mediatech, Inc.) supplemented with 10% pooled human platelet lysate (pHPL, kindly provided by Dr. Dirk Strunk, Department of Hematology and Stem Cell Transplantation, Medical University of Graz, Austria), 1 supplemented with 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (Sigma-Aldrich). The BM mononuclear cell content was analyzed by automated blood count (Beckman Coulter), and mononuclear cells were seeded at a density of $5 \times 10^4$ cells/cm$^2$ in tissue-culture flasks and cultured at 37°C in 5% CO$_2$ incubator. The non-adherent cells were removed by completely changing the medium after 3 days, and the adherent cells were continuously cultured. The cultures were fed twice weekly by replacing 30% of the medium with fresh supplemented medium. The cells were harvested before reaching confluence by applying 0.25% trypsin and 1 mM EDTA (Life Technologies). MSC were cryopreserved and early passage (passage 2-3) samples were used for study. As observed in our previous studies, isolated MSC are CD73+/CD90+/CD105+ (see references 4 and 14 in the manuscript).

Flow Cytometry

Flow cytometry to assess standard MSC lineage markers on MSC were performed using antibodies against CD73-PE (PE; BD), CD105-PE (PE; eBioscience) and CD90 (APC-Cy7;
Beckman Coulter). Cells were analyzed on LSR-II flow cytometer and the data was analyzed using FlowJo software.

**Immunoblot analysis**

Cells were lysed and protein transferred to a membrane and western blotting analysis performed with antibodies against p53, p21 (both from Santa Cruz Biotechnology, Dallas, TX) and Tubulin (Sigma-Aldrich). Signals were detected by using the Odyssey Infrared Imaging System and quantitated by Odyssey software version 3.0 (both LI-COR Biosciences, Lincoln, NE). Tubulin was used as a loading control.

**Gene and miR expression analysis**

Real-time PCR (qRT-PCR) was conducted using an ABI 7900HT Fast Real-Time PCR System (Life Technologies). We ran duplicate 20 μl reactions containing the equivalent of 1 ng total RNA. We used the following TaqMan Gene Expression Assays (Life Technologies) as directed by the manufacturer: p53 (TP53; Hs01034249_m1), BCL-X_L (BCL2L1; Hs00236329_m1), TP53INP1 (Hs01003820_m1), BBC3 (Hs00248075_m1), CDKN1A (Hs00355782_m1), CCND1 (Hs00765553_m1), and 18S (Hs03928985_g1). For miR analysis, Taqman assays for miR-93 (000432) and U6 snRNA (#001973) were used. We used RQ Manager 1.2.1 (Life Technologies) to analyze the data.

**Statistical analyses**

For RPPA, supercurve algorithms were used to generate a single value from the five serial dilutions. Loading control and topographical normalization procedures accounted for protein
concentration and background staining variations.\textsuperscript{28-31} Analysis using unbiased hierarchical clustering perturbation bootstrap clustering, and principle component analysis was then done as fully described in a previous publication using available R packages and Qlucore software (Version 3.1, Qlucore Inc. Lund Sweden).\textsuperscript{28-31} Comparison of the protein levels between paired samples was done by performing paired $t$ test. Association between protein expression levels and categorical clinical variables were assessed in R using standard $t$ tests, linear regression, or mixed effects linear models. Unbiased hierarchical clustering was performed using the weighted average method and the associated figures show expression normalized to median = 0, variance = 1. The $P$-value and the associated Q-value (a measure of the false discovery rate) are shown for each clustering analysis. Association between continuous variable and protein levels were assessed by using the Pearson and Spearman correlation and linear regression. Bonferroni corrections were done to account for multiple statistical parameters for calculating statistical significance.
Supplemental Figure 1. AML MSC and NL MSC express MSC lineage markers. Flow cytometry on three representative Normal MSC and three representative AML MSC was performed using CD73, CD90, and CD105 antibodies. Data was analyzed by FlowJo.
Supplemental Figure 2. Protein expression is distinct between MSC and blood cells. PCA (A) and heat map (B) of hierarchical clustering of 151 proteins examined in AML MSC (blue), NL MSC (light blue), AML blasts (pink) and normal CD34+ cells (green) is depicted.
Supplemental Figure 3. Validation of expression of proteins that are elevated in AML MSC as identified by RPPA. Protein was isolated from AML derived MSC (n = 3). Immunoblot analysis performed with antibodies against STAT1, PDK1, CK2, CCND1, GSK3 A/B, SPP1, CDK4, p53, BCL-XL, ITGA2, NOTCH 1, PPP2R2A, STAT5A/B, DIABLO, p21, PARP1, and BAK1. Tubulin was included as control for each filter analyzed. Images were obtained using LiCor imager.
Supplemental Figure 4. Expression of various proteins are different in normal versus AML MSC across different age groups. (A) Protein expression as determined by RPPA for BCL-X\textsubscript{L} and PPP2R2A/B/C/D are compared among AML patients and healthy individuals in age groups of under 30 years old (left), 30-49 years old (center) and 50-59 years old (right). Statistical analysis was performed as described in “Methods”. (B) Protein was isolated from normal donor MSC (n = 3) and AML derived MSC (n = 3) from individuals 40-49 years old. Immunoblot analysis performed with antibodies against phosphorylated ERK, MCL-1, p53, and Tubulin. Ratio of protein expression to Tubulin loading control was determined by densitometry using LiCor imager.
Supplemental Figure 5. STAT5 expression is differentially correlated with STAT1 and hnRPK in normal and AML MSC. Pearson correlation of protein expression as determined by RPPA with STAT5 demonstrates differences between normal (A) and AML (B) MSC. Reverse correlations are found with STAT1 (green) and hnRPK (black).
Supplemental Figure 6. Ingenuity Pathway Analysis Reveals Proteins Identified by Unbiased Clustering in Group 3 Proteins (Elevated in Normal MSC) Are Involved in Adipogenesis. Signature Proteins Are Denoted In Pink.
Supplemental Figure 7. Ingenuity Pathway Analysis Reveals Proteins Identified by Unbiased Clustering in Group 1 and Group 2 (Elevated in AML MSC) Are Involved in In PI3K/AKT Signaling (Right). Signature Proteins Are Denoted In Pink.
Supplemental Figure 8. Ingenuity Pathway Analysis (Software from Qiagen) Reveals Proteins Identified by Unbiased Clustering in salvage versus newly diagnosed MSC from AML patients Are Involved In osteoblast differentiation. Signature Proteins Are Denoted In Pink.
Supplemental Figure 9. Gene expression in AML and normal MSC. RNA was isolated from AML MSC (n = 10) and normal MSC (n = 9) and qRT-PCR performed to measure expression of BCL2L1, CCND1; PPP2R2A, and CDKN1A. Expression was normalized to 18S.
| Protein     | Description 1 | Description 2 |
|-------------|---------------|---------------|
| AKT         | FOXO1p24.FOXO3p32 | PA2G4.pT37.p46 |
| AKT1        | FOXO3         | PA2G4.pT70    |
| AKT1.2.3.p308 | FOXO3.p318.321 | PARK7         |
| AKT1.2.3.p473 | GAB2          | PARP1         |
| AKT1S1      | GAB2.p452     | PDK1          |
| AKT1S1.p    | GAPDH         | PDK1.p241     |
| AKT2        | GATA3         | PECAM1        |
| AKT3        | GSKA,B        | PPARG         |
| ARC         | GSKA.B.p21.9  | PPP2R2A/B/C/D |
| ATF3        | HDAC3         | PRKAA1.2      |
| BAD         | HIF1A         | PRKAA1.2.p172 |
| BAD.p112    | HNRNPK        | PSMD9         |
| BAD.p136    | HSP90AA1.B1   | PSMD9.1       |
| BAD.p155    | HSPA1A.L      | PTEN          |
| BAK1        | HSPB1         | PTGS2         |
| BAX         | INPP5D        | PTK2          |
| BCL2        | IRS1.phospho.ser.1101 | RAC1.2.3 |
| BCL2L1      | ITGA2         | RELA          |
| BCL2L11     | ITGAL         | RPS6          |
| BECN1       | ITGB3         | RPS6.p235.236 |
| BID         | JMJD6         | RPS6.p240.244 |
| BIRC5       | JUN.p73       | RPS6KB1       |
| CAV1        | JUNB          | RPS6KB1.ph389 |
| CCNB1       | KDR           | SFN           |
| CCND1       | KIT           | SIRT1         |
| CCND3       | LCK           | SMAD1         |
| CCNE1       | LEF1          | SMAD4         |
| CD34        | LGALS3        | SMAD6         |
| CDK1        | LYN           | SPP1          |
| CDK2        | MAP2K1        | SQSTM0        |
| CDK4        | MAP2K1.2.p217.221 | SRC          |
| CDKN1A      | MAPK1         | SRC.p416      |
| CREB1       | MAPK1.3.p202.204 | SRC.p527    |
| CREB1.p133  | MAPK14        | STAT1         |
| CSNK2A1     | MAPK14.1      | STAT1.p701    |
| CTNNA1      | MAPK8         | STAT3         |
| CTNNB1      | MAPK9         | STAT3.p705    |
| CTNNB1.p33.37.41 | MCL1         | STAT3.p727    |
| DIABLO      | MDM2          | STAT5A.B      |
| EGFR        | MS4A1         | STAT5A.B.p694 |
| EGFR.p992   | MSI2          | STK11         |
| Gene 1       | Gene 2       | Gene 3       |
|-------------|-------------|-------------|
| EGLN1       | MTOR        | STMN1       |
| EIF2S1      | MTOR.p2448  | TCF4        |
| EIF2S1.p51. | MYC         | TGM2        |
| EIF4E       | NOTCH1.cl1744 | TP53      |
| ELK1.p383   | NOTCH3      | TP53.1      |
| ERBB2       | NPM1.1      | TSC2        |
| ERBB2.p1248 | NR4A1       | VHL         |
| ERG         | NRP1        | XIAP        |
| FN1         | PA2G4       | YWHAE       |
|             |             | YWHAZ       |
Supplemental Table 2: Expression of proteins as determined by RPPA in AML MSC and normal MSC according to age. Statistically significant (p < 0.05) values are in **bold**. An * follows values with p > 0.05.

### Elevated AML-MSC

| Proteins                          | p value Age < 30 | p value Age 31-49 | p value Age 50-59 |
|-----------------------------------|------------------|------------------|------------------|
| n =18                             | n =13            | n =17            | n =20            |
| Statistically significant in all groups n =2 |                  |                  |                  |
| BCL2L1 (BCL-XL)                   | **0.015**        | **0.005**        | **0.033**        |
| PPP2R2A/B/C/D                     | **0.006**        | **0.002**        | **0.015**        |
| Statistically significant in two groups (n = 8) |                  |                  |                  |
| CSN2KA1                           | **0.020**        | 0.063*           | **0.002**        |
| STAT5A/B                          | **0.002**        | **0.022**        | 0.068*           |
| TP53                              | **0.005**        | 0.288*           | **0.001**        |
| CDKN1A (p21)                      | **0.011**        | **0.011**        | 0.715*           |
| CDK4                              | 0.001            | 0.042            | 0.715*           |
| ERBB2                             | **0.025**        | **0.013**        | 0.503*           |
| GSK3A/B                           | **0.048**        | 0.121*           | **0.015**        |
| STAT1                             | **0.025**        | **0.001**        | 0.670*           |
| Statistically significant in one group (n = 6) |                  |                  |                  |
| p-PDK1 (S241)                     | **0.007**        | 0.079*           | 0.361*           |
| ITGA2                             | 0.154*           | **0.002**        | 0.224*           |
| PARP1                             | 0.799*           | **0.010**        | 0.162*           |
| CCND1                             | 0.160*           | **0.001**        | 0.855*           |
| BAK1                              | 0.109*           | 0.084*           | **0.029**        |
| SPP1                              | 0.800            | **0.017**        | 0.334            |
| Statistically significant in no groups (n = 2) |                  |                  |                  |
| NOTCH (cleaved 1744)              | 0.228*           | 0.079*           | 0.761*           |
| DIABLO                            | 0.109*           | 0.196*           | 0.144*           |

### Elevated NL MSC

| Proteins                          | p value Age < 30 | p value Age 31-49 | p value Age 50-59 |
|-----------------------------------|------------------|------------------|------------------|
| n =7                              | n =37            | n =25            | n =6             |
|                                  |                  |                  |                  |

15
| Protein       | Group 1 Mean  | Group 2 Mean | p-value |
|---------------|--------------|--------------|---------|
| SMAD4         | 0.028        | 0.014        | 0.144*  |
| STMN1         | 0.021        | 0.001        | 0.543*  |
| EIF2S1        | 0.041        | 0.010        | 0.162*  |
| SMAD1         | 0.370*       | 0.003        | 0.626*  |
| SIRT1         | 0.099*       | 0.013        | 0.543*  |
| p-Foxo1/3 (S32)| 0.167*   | 0.001        | 0.465*  |

Statistically significant in all groups (n = 0)

Statistically significant in two groups (n = 2)

Statistically significant in one group (n = 4)

Statistically significant in no groups (n = 1)

HSP90AA1/B1   0.083*        0.247*      0.503*