SUPPLEMENTARY APPENDIX

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

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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 5×10^4 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

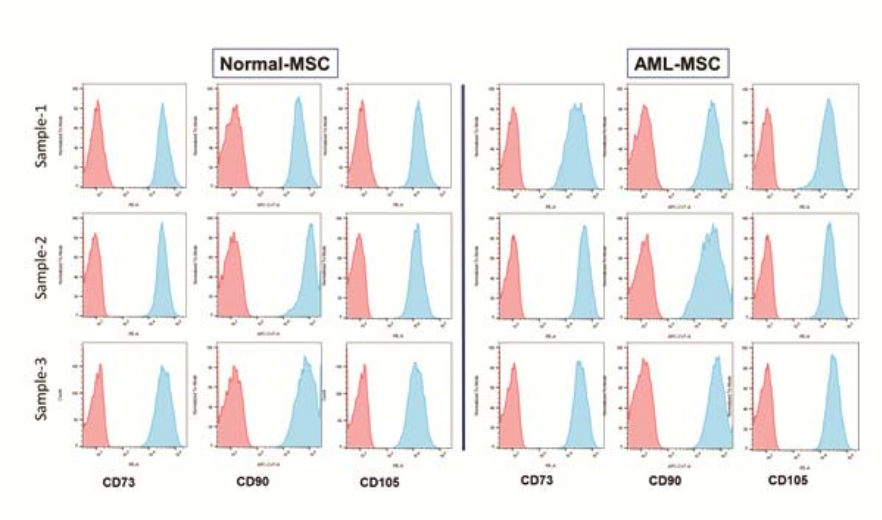
Real-time PCR (qRT-PCR) was conducted using an ABI 7900HT Fast Real-Time PCR System (Life Technologies). We ran duplicate 20 µl reactions containing the equivalent of 1 ng total RNA). We used the following TaqMan Gene Expression Assays (Life Technologies) as directed by the manufacturer: p53 (TP53; Hs01034249_m1), 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

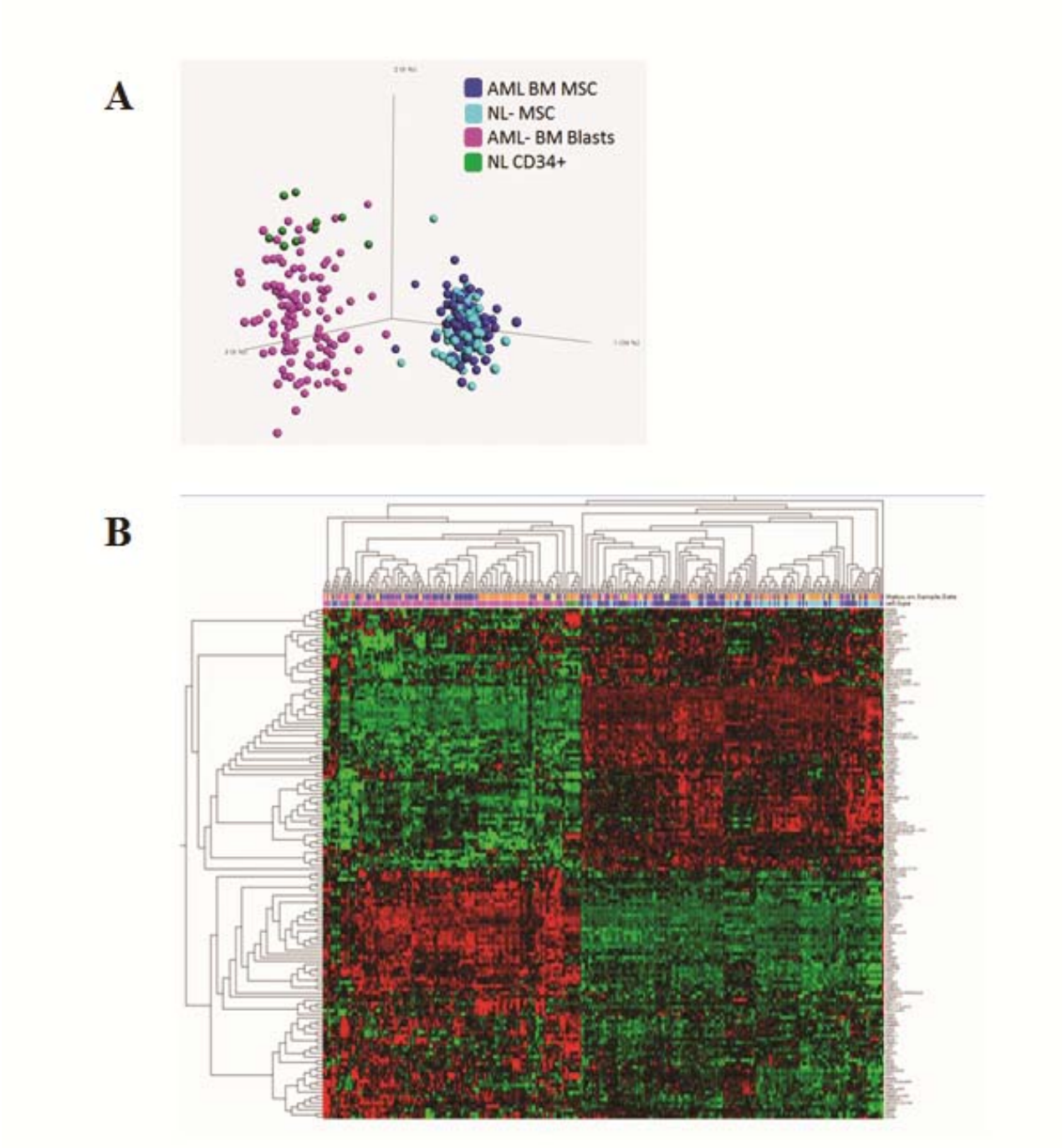
For RPPA, supercurve algorithms were used to generate a single value from the five serial dilutions.[28-31] Loading control and topographical normalization procedures accounted for protein

concentration and background staining variations.[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).[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.

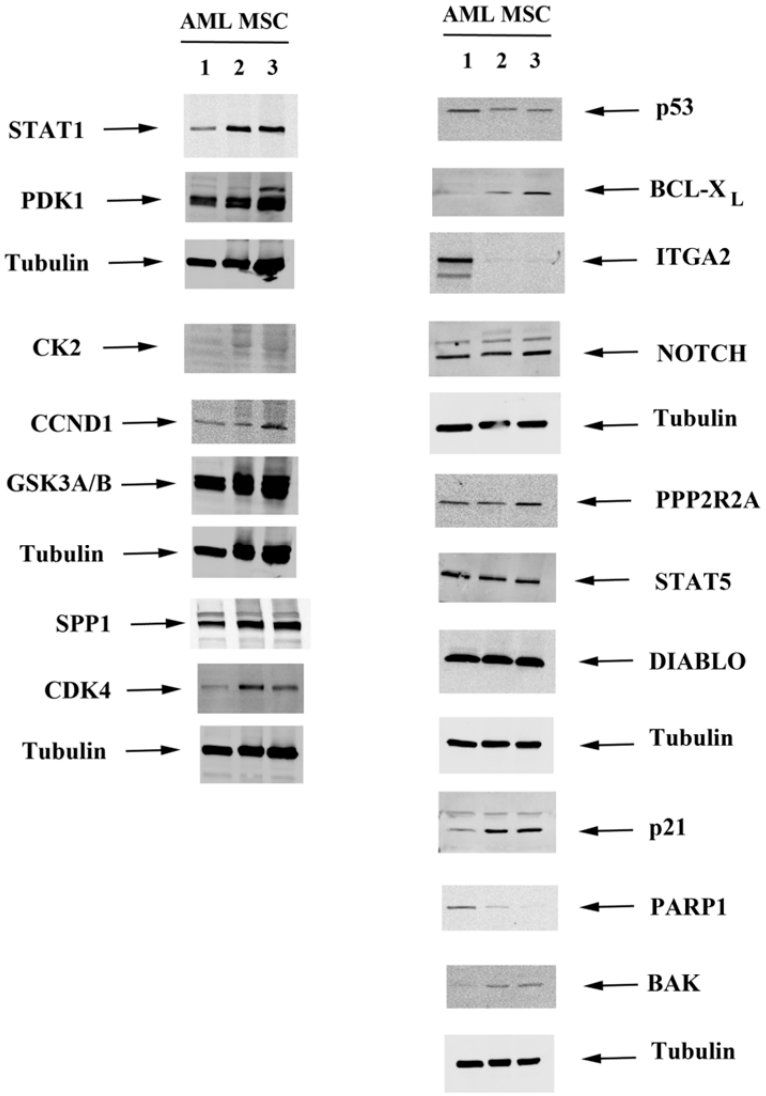
Supplemental Figures



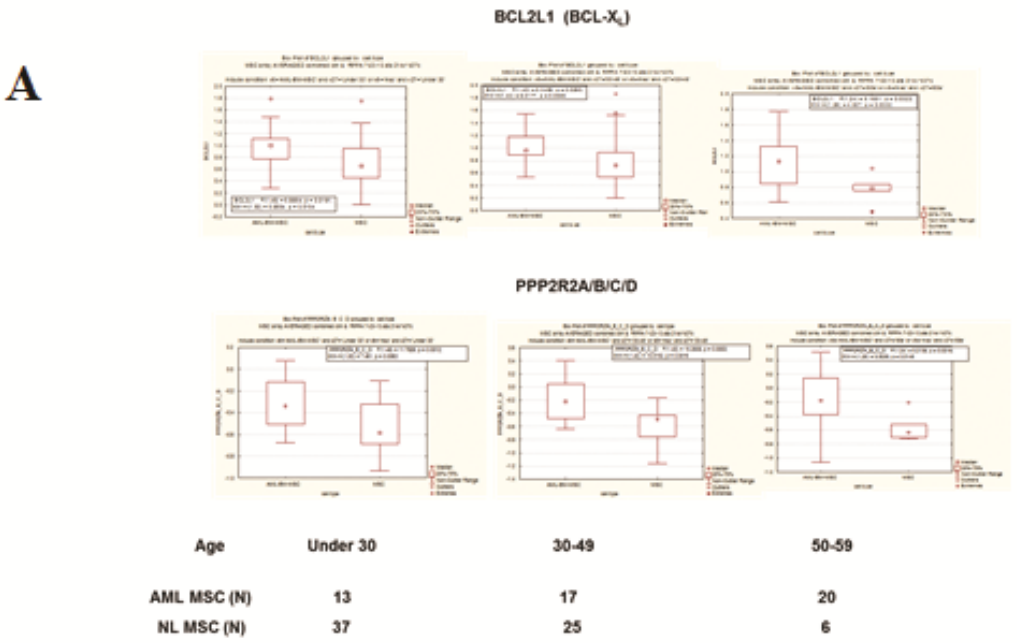
Supplemental Figure 1. AML MSC and NL MSC express MSC lineage markers. Flow cytometry on three representative Normal MSC and three representative AML MSC was performed using CD73, CD90, and CD105 antibodies. Data was analyzed by FlowJo.

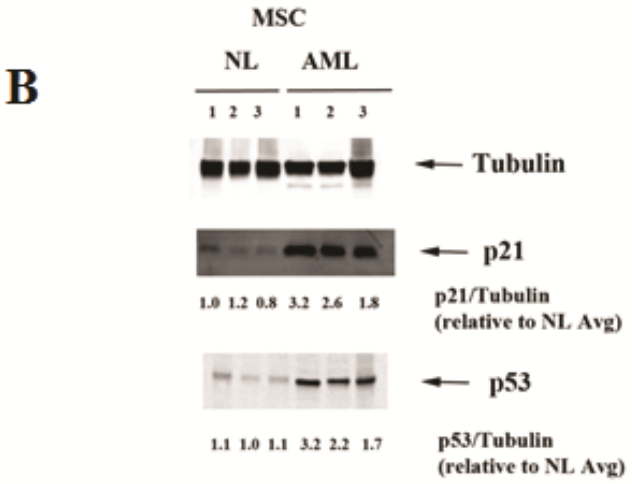


Supplemental Figure 2. Protein expression is distinct between MSC and blood cells. PCA (A) and heat map (B) of hierarchical clustering of 151 proteins examined in AML MSC (blue), NL MSC (light blue), AML blasts (pink) and normal CD34+ cells (green) is depicted.

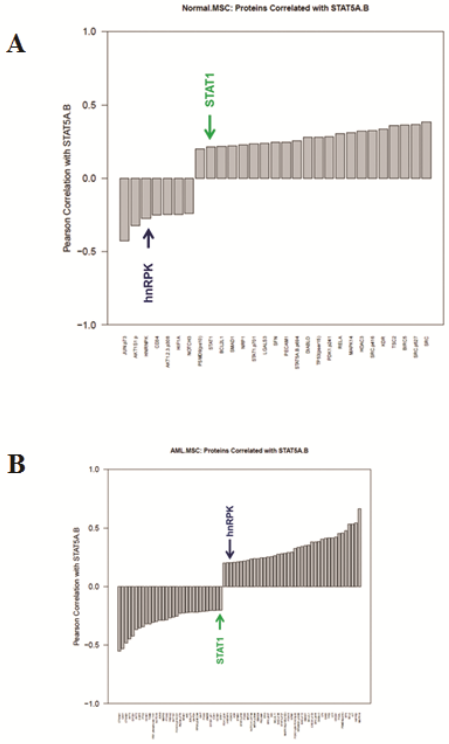


Supplemental Figure 3. Validation of expression of proteins that are elevated in AML MSC as identified by RPPA. Protein was isolated from AML derived MSC (n = 3). Immunoblot analysis performed with antibodies against STAT1, PDK1, CK2, CCND1, GSK3 A/B, SPP1, CDK4, p53, BCL-X_L, ITGA2, NOTCH 1, PPP2R2A, STAT5A/B, DIABLO, p21, PARP1, and BAK1. Tubulin was included as control for each filter analyzed. Images were obtained using LiCor imager.

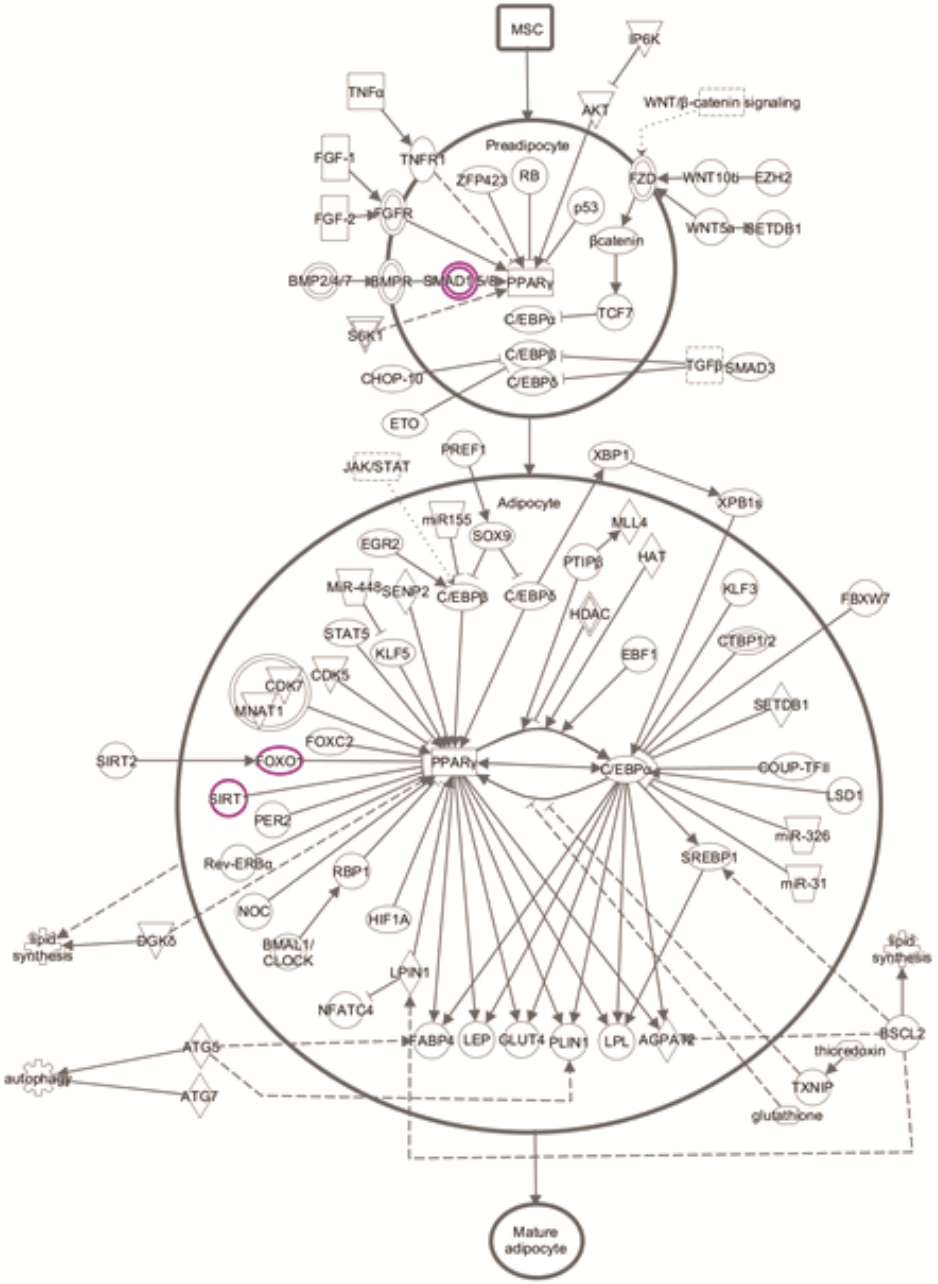




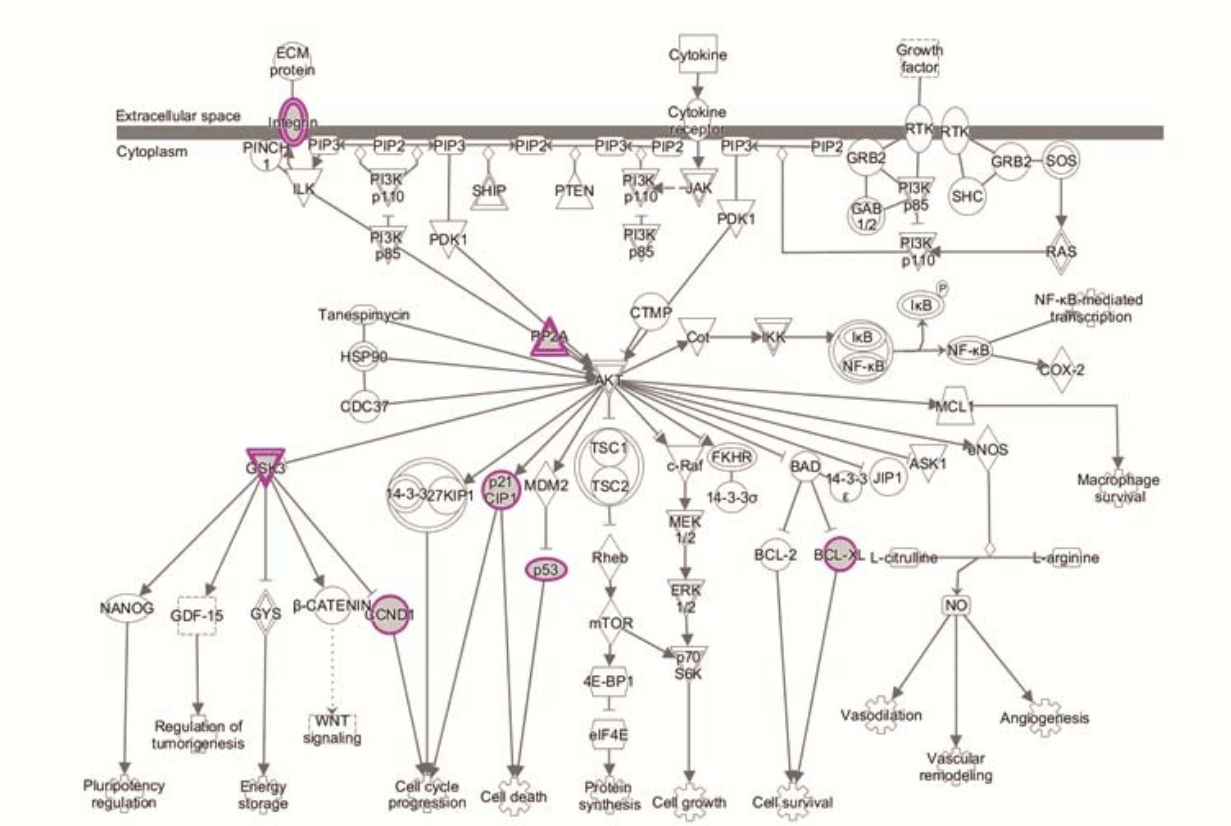
Supplemental Figure 4. Expression of various protiens are different in normal versus AML MSC across different age groups. (A) Protein expression as determined by RPPA for BCL-X_L and PPP2R2A/B/C/D are compared among AML patients and healthy individuals in age groups of under 30 years old (left), 30-49 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 individulas 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.



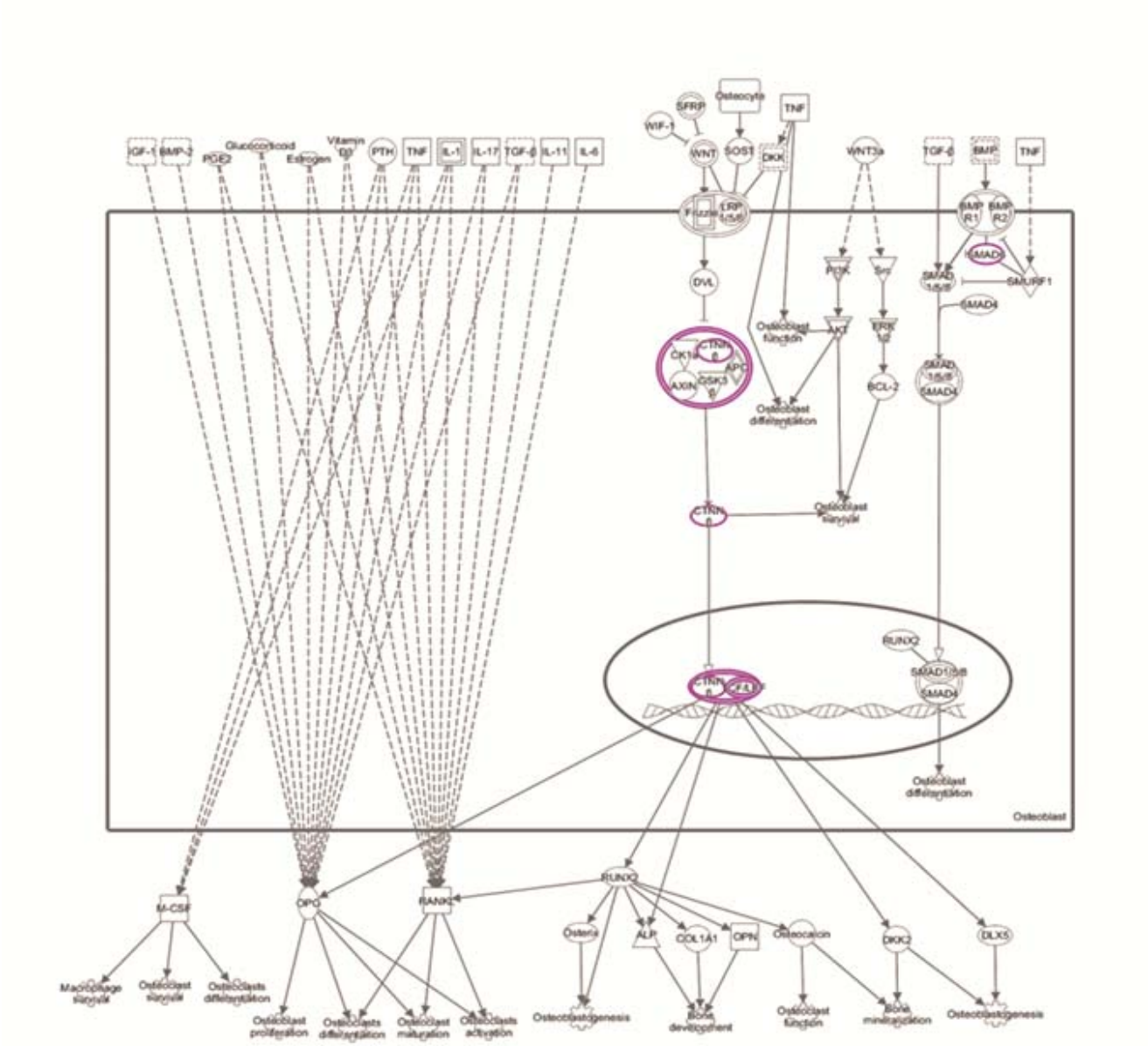
Supplemental Figure 5. STAT5 expression is differentially correlated with STAT1 and hnRPK in normal and AML MSC. Pearson correlation of protein expression as determined by RPPA with STAT5 demonstrates differences between normal (A) and AML (B) MSC. Reverse coorelations are found with STAT1 (green) and hnRPK (black).



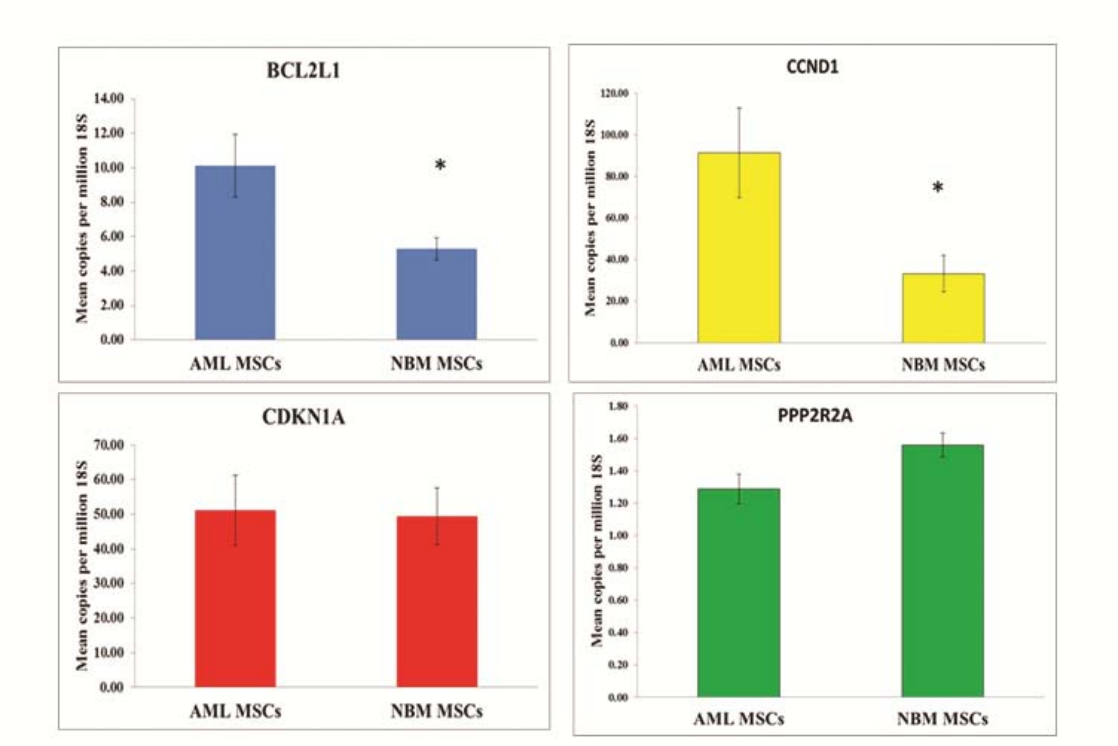
Supplemental Figure 6. Ingenuity Pathway Analysis Reveals Proteins Identified by Unbiased Clustering in Group 3 Proteins (Elevated in Normal MSC) Are Involved in Adipogenesis. Signature Proteins Are Denoted In Pink.



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.

144 Supplemental Table 1: List of proteins analyzed by RPPA.

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

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 n =18	p value Age < 30 n =13	p value Age 31-49 n =17	p value Age 50-59 n =20
Statistically significant in all groups n =2			
BCL2L1 (BCL-X_L)	**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 n =7	p value Age < 30 n =37	p value Age 31-49 n =25	p value Age 50-59 n =6

Statistically significant in all groups n = 0			
Statistically significant in two groups (n = 2)			
SMAD4	**0.028**	**0.014**	0.144*
STMN1	**0.021**	**0.001**	0.543*
Statistically significant in one group (n = 4)			
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 no groups (n = 1)			
HSP90AA1/B1	0.083*	0.247*	0.503*

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