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,[1] Teresa McQueen,[1] YiHua Qui,[1] Zhihong Zeng,[1] Sherry Pierce,[1] Rodrigo Jacamo,[1] Suk-Young Yoo,[3] Phuong M. Le,[1] Jeffrey Sun,[1] Numsen Hail Jr,[1] Marina Konopleva[1] and Michael Andreeff[1]

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

*SMK and PPR contributed equally to this work.

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Correspondence:

skornblau@mdanderson.org or
mandreef@mdanderson.org

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ABSTRACT

Mesenchymal stromal cells (MSC) support acute myeloid leukemia (AML) cell survival in the bone marrow (BM) microenvironment. Protein expression profiles of AML-derived MSC are unknown. Reverse phase protein array analysis was performed to compare expression of 151 proteins from AML-MSC (n=106) with MSC from healthy donors (n=71). Protein expression differed significantly between the two groups with 19 proteins over-expressed in leukemia stromal cells and 9 over-expressed in normal stromal cells. Unbiased hierarchical clustering analysis of the samples using these 28 proteins revealed three protein constellations whose variation in expression defined four MSC protein expression signatures: Class 1, Class 2, Class 3, and Class 4. These cell populations appear to have clinical relevance. Specifically, patients with Class 3 cells have longer survival and remission duration compared to other groups. Comparison of leukemia MSC at first diagnosis with those obtained at salvage (i.e. relapse/refractory) showed differential expression of 9 proteins reflecting a shift toward osteogenic differentiation. Leukemia MSC are more senescent compared to their normal counterparts, possibly due to the over-expressed p53/p21 axis as confirmed by high β-galactosidase staining. In addition, overexpression of BCL-X_L in leukemia MSC might give survival advantage under conditions of senescence or stress and over-expressed galectin-3 exerts profound immunosuppression. Together, our findings suggest that the identification of specific populations of MSC in AML patients may be an important determinant of therapeutic response.

Introduction

There is growing evidence to support the importance of the leukemia bone marrow (BM) niche in the process of acute myeloid leukemia (AML) chemoresistance.[1,2] Hence, optimal therapeutic strategies should also address neighboring cells in the tumor microenvironment. The critical support cells in the leukemia BM microenvironment are mesenchymal stromal cells (MSC).[3-8] Depending on the type, MSC can act either to support or suppress tumors.[4,8-15] Our group and others have found that MSC support leukemia cell survival by diverse mechanisms that include secretion of cytokines and chemokines, activation of survival signaling in tumor cells, and blocking immune surveillance by suppressing natural killer (NK) and T cells.[2-5,13]

Mesenchymal stromal cells are essential for human hematopoiesis, particularly as a source of SDF-1, which regulates homing, proliferation, and differentiation.[6,9,10,16-18] Moreover, studies from our group and others have demonstrated that MSC protect leukemia cells from chemotherapy.[6,19-23] We have recently found that there is reciprocal activation of NFkB signaling between MSC and AML and acute lymphoblastic leukemia (ALL) cells that likely contribute to the effectiveness of the microenvironment to protect malignant cells.[7] Medyouf *et al.* recently demonstrat-

ed that blast cells from myelodysplastic syndrome (MDS) patients induce changes in MSC reflecting reprograming of the stromal cells.[24] MSC may also influence hematopoietic precursors to promote leukemogenesis as evidenced by the development of AML and MDS in mice where the MSC osteo-progenitors were engineered to lack Dicer, a key regulator of microRNA (miR) processing.[2] Furthermore, a recent study from Zhao *et al*. reported that p21 could be critical for inducing senescence in MSC from MDS patients with concomitant induction of interleukin-6 (IL6) and transforming growth factor β (TGF-β).[25] This study is consistent with findings that support the role of Dicer in regulating MSC biology and also establish a possible mechanism of aberrant survival functions in malignant MSC that may be associated with p21 and senescence. The ability of malignant MSC to withstand senescence may depend on the expression of the anti-apoptotic molecule BCL-X_L.[26,27]

The cellular composition of stromal cells in a cancer microenvironment, such as the leukemic BM niche, is likely markedly different from that of the normal BM. We, therefore, set out to study the protein expression and activation in leukemic MSC (AML-MSC) and compared and contracted these to normal MSC (NL-MSC) to determine if and how they are functionally different. Reverse phase protein array analysis (RPPA, pioneered in our laboratory)[28-32] was used to examine expression of 151 proteins in MSC derived from AML BM (n=106) with those derived from healthy donors (n=71). The results presented here identify 28 that were differentially expressed between the two. Importantly, the 28 proteins identified as differentially expressed in the AML *versus* normal MSC could be grouped into four protein constellation (PC) expression signatures with different biological properties and clinical implications regarding patient response to therapy.

Methods

Patients' samples

Bone marrow was obtained from AML patients (n=106) undergoing diagnostic BM aspiration and from healthy donors (n=71) who were undergoing BM harvest for use in allogeneic BM transplantation. Samples were acquired in accordance with the regulations and protocols approved by the Investigational Review Board of MD Anderson Cancer Center. Informed consent was obtained in accordance with the Declaration of Helsinki. Samples were analyzed under an Institutional Review Board-approved laboratory protocol. Patients' characteristics are presented in Table 1. Details of isolation of MSC are available in the *Online Supplementary Methods.*

RPPA

Proteomic profiling was carried out on MSC samples from patients with AML and healthy donors using RPPA. The RPPA method and sample validation technique are described fully elsewhere.[28-32] Antibodies against 151 proteins were used for analysis. (A list and the source of the antibodies and the concentrations utilized is provided in the *Online Supplementary Table S1*). The sources of antibodies have been been reported previously.[30] An IgG subtype-specific secondary antibody was used to amplify the signal, and finally a stable dye was precipitated. The stained slides were analyzed using the Microvigene software (version 3.0, Vigene Tech, Carlise, MA, USA) to produce quantified data. Statistical analyses are described in the *Online Supplementary Methods.*

Cell senescence assessment

Microscopy assessment of β-galactosidase staining was used to detect cell senescence using a detection kit from Cell Signal Technology (Boston, MA, USA). Early passage cells (passage 2) were imaged using a Nikon Coolpix 950 camera attached to a Nikon TMS light microscope (Nikon Instruments Inc.). AML-MSC (n=4) and NL-MSC (n=5) were lysed in kit buffer. Measurement of β-galactosidase was performed using an *in vitro* fluorometric assay with fluorescein di-β-D-galactopyranoside (FDG) as substrate. Incubation time was 2 hours (h). Fluorescence was measured using an Optima Fluorometer (Durham, NC, USA). Activity is presented as fluorescence units/1000 cells/minute.

Pathway analysis

String software (String 10.1; available from: *http://string-db.org*)[33] was used to determine protein associations. Pathway analysis to identify canonical pathways, upstream regulators, and protein networks was performed using Ingenuity Pathway software (Qiagen).

Results

Proteins are differentially expressed in AML *versus* healthy MSC

We have routinely utilized RPPA to analyze protein expression from clinical samples from many hematologic malignancies.[28-32] We examined protein expression in blasts from newly diagnosed AML patients (n=85), $CD34^+$ cells from normal donors (n=10), MSC from healthy donors (n=71), and MSC from newly diagnosed AML patients (n=54). Both normal MSC and AML-MSC expressed MSC defining lineage markers CD73, CD90, and CD 105 as determined by flow cytometry (*Online Supplementary Figure S1*). MSC from salvage samples (i.e. relapse/refractory) were also studied (n=46). The RPPA was probed with 151 antibodies targeting 119 different proteins (114 targeting total protein with 32 paired antibodies targeting phosphoepitopes on 26 proteins, and 5 with only a phosphoepitope but not total protein epitope) covering a wide variety of cellular functions and pathways (*Online Supplementary Table S1*). Protein expression in AML-MSC, NL-MSC, AML blasts and normal $CD34^+$ cells was compared using principle component analysis (*Online Supplementary Figure S1*) and unbiased hierarchical clustering (*Online Supplementary Figure S2B*). NL-MSC and AML-MSC formed a cluster distinct from AML blasts and NL-$CD34^+$ cells with the vast majority of the 151 proteins tested showing statistically significant differential expression (143 of 151, P=0.01; 124 of 151, $P \leq 10^{-6}$) between MSC and the blast/$CD34^+$ cells. This unsurprising observation is consistent with a previous report that gene expression profiles are distinct between blood cells and MSC.[34] Principal component analysis (PCA) also shows that protein expression in NL-$CD34^+$ cells is distinct from that of AML blasts. These findings were identical to those observed when the analysis was restricted to samples from newly diagnosed patients alone.

Next we investigated whether protein expression in AML-MSC was different from that of NL-MSC. In PCA, the NL-MSC occupied a distinct space from that of the AML-MSC (Figure 1A). Unbiased hierarchical clustering comparing AML-MSC and NL-MSC revealed differential expression of 28 of those proteins (P<0.001; Q=0.0059). The Q-value, a measure of the false discovery rate determined by a β-uniform mixture model highlights that these

differences are very unlikely to be random and suggest that there are significant differences in the protein expression patterns of AML-MSC relative to those of NL-MSC. The proteins clustered into three PCs (from top to bottom in Figure 1B). Nineteen of these proteins had generally higher expression in AML-MSC, including P1 [STAT1, p-

Table 1. Patients' demographics (see Figures 2A and 3A).

Variable	Category	Total	Class 2	Class 1	Class 3	Class 4	*P*
Cohort size	Number	101	6	13	18	64	
	Percentage	100.00%	5.90%	12.90%	17.80%	63.40%	
Status	New	53.50%	66.70%	53.80%	66.70%	48.40%	0.55
	Salvage	45.50%	33.30%	46.20%	33.30%	50.00%	
Sex	M	61.40%	33.30%	30.80%	72.20%	67.20%	**0.03**
	F	38.60%	66.70%	69.20%	27.80%	32.80%	
Race	Asian	6.90%	0.00%	7.70%	11.10%	6.30%	0.5
	Black	7.90%	16.70%	15.40%	0.00%	7.80%	
	White	73.30%	50.00%	69.20%	83.30%	73.40%	
	Hispanic	9.90%	33.30%	7.70%	5.60%	9.40%	
	Unknown	3.00%	0.00%	7.70%	0.00%	3.10%	
FAB	M0	4.00%	0.00%	0.00%	5.60%	4.70%	0.21
	M1	16.80%	16.70%	38.50%	5.60%	15.60%	
	M2	24.80%	16.70%	23.10%	22.20%	26.60%	
	M4	26.70%	66.70%	23.10%	27.80%	23.40%	
	M5	12.90%	0.00%	7.70%	16.70%	14.10%	
	M6	3.00%	0.00%	0.00%	11.10%	1.60%	
	RAEB-T	4.00%	0.00%	0.00%	0.00%	6.30%	
	Unk	7.90%	0.00%	7.70%	11.10%	7.80%	
PS	Asymptomatic	15.80%	16.70%	15.40%	11.10%	17.20%	0.44
	Symptoms	62.40%	50.00%	46.20%	77.80%	62.50%	
	In bed <50	13.90%	33.30%	23.10%	5.60%	12.50%	
	In bed >50	2.00%	0.00%	7.70%	0.00%	1.60%	
	100% bedridden	1.00%	0.00%	0.00%	5.60%	0.00%	
AHD	N	75.20%	50.00%	84.60%	83.30%	73.40%	0.33
	Y	24.80%	50.00%	15.40%	16.70%	26.60%	
Cyto	Favorable	2.00%	16.70%	7.70%	0.00%	0.00%	**0.026**
	Intermediate	54.50%	83.30%	53.80%	50.00%	53.10%	
	Unfavorable	43.60%	0.00%	38.50%	50.00%	46.90%	
FLT3	Negative	65.30%	33.30%	69.20%	66.70%	64.10%	0.0979
	Positive	20.80%	16.70%	23.10%	16.70%	21.90%	
	Not Done	8.90%	50.00%	7.70%	16.70%	14.10%	
D835	Negative	79.20%	50.00%	92.30%	77.80%	76.60%	**0.005**
	Positive	6.90%	0.00%	0.00%	5.60%	9.40%	
	Not Done	8.90%	50.00%	7.70%	16.70%	14.10%	
NPM1	Negative	46.50%	50.00%	69.20%	72.20%	45.30%	0.08
	Positive	10.90%	0.00%	7.70%	0.00%	17.20%	
	Not Done	5.00%	50.00%	23.10%	27.80%	37.50%	
Response	CR	58.80%	66.70%	71.40%	63.60%	53.30%	0.8
	PR	29.70%	0.00%	0.00%	0.00%	3.30%	
	HI	1.00%	0.00%	0.00%	9.10%	6.70%	
	No response	3.00%	33.30%	14.30%	9.10%	13.30%	
	FAIL	6.90%	0.00%	14.30%	18.20%	23.30%	
Relapse	Yes	70.00%	100.00%	80.00%	28.60%	81.30%	0.076
Vital	Alive	21.60%	0.00%	28.60%	36.40%	16.70%	0.41
	Dead	78.40%	100.00%	71.40%	63.60%	83.30%	

M: male; F: female; FAB: French-American-British Classification; PS: propensity score; AHD: antecedent hematologic disease; N: no; Y: yes; Cyto: cytogenetic profile; CR: complete response; PR: partial response; HI: hematologic improvement; *Statistically significant (*P*) sets are in bold.

PDK1 (S241)], CCND1, CDKN1A (p21), ITGA2, PARP1, PPP2R2A/B/C/D, the PP2A B regulatory subunit family B55, BAK1, CSNK2A1, CDK4, GSK3A/B) and PC2 [STAT5A/B, BCL2L1 (BCL-XL)], DIABLO, TP53 (p53), NOTCH 1 (cleaved 1744), SPP1, p-EGFR (Y992), and ERBB2). Expression of 17 of the 19 proteins was validated by immunoblot analysis (*Online Supplementary Figure S3*). Although expression of EGFR and ERBB2 expression could not be confirmed by western blot analysis, this may reflect the enhanced sensitivity of RPPA over standard immunoblot technology. The remaining nine proteins in PC3 were elevated in healthy donor MSC compared to AML-MSC: SMAD1, CREB1.p133 STMN1, SIRT1, CREB1 SMAD4, p-Foxo1/3 (S32), HSP90AA1/B1, and EIF2S1.

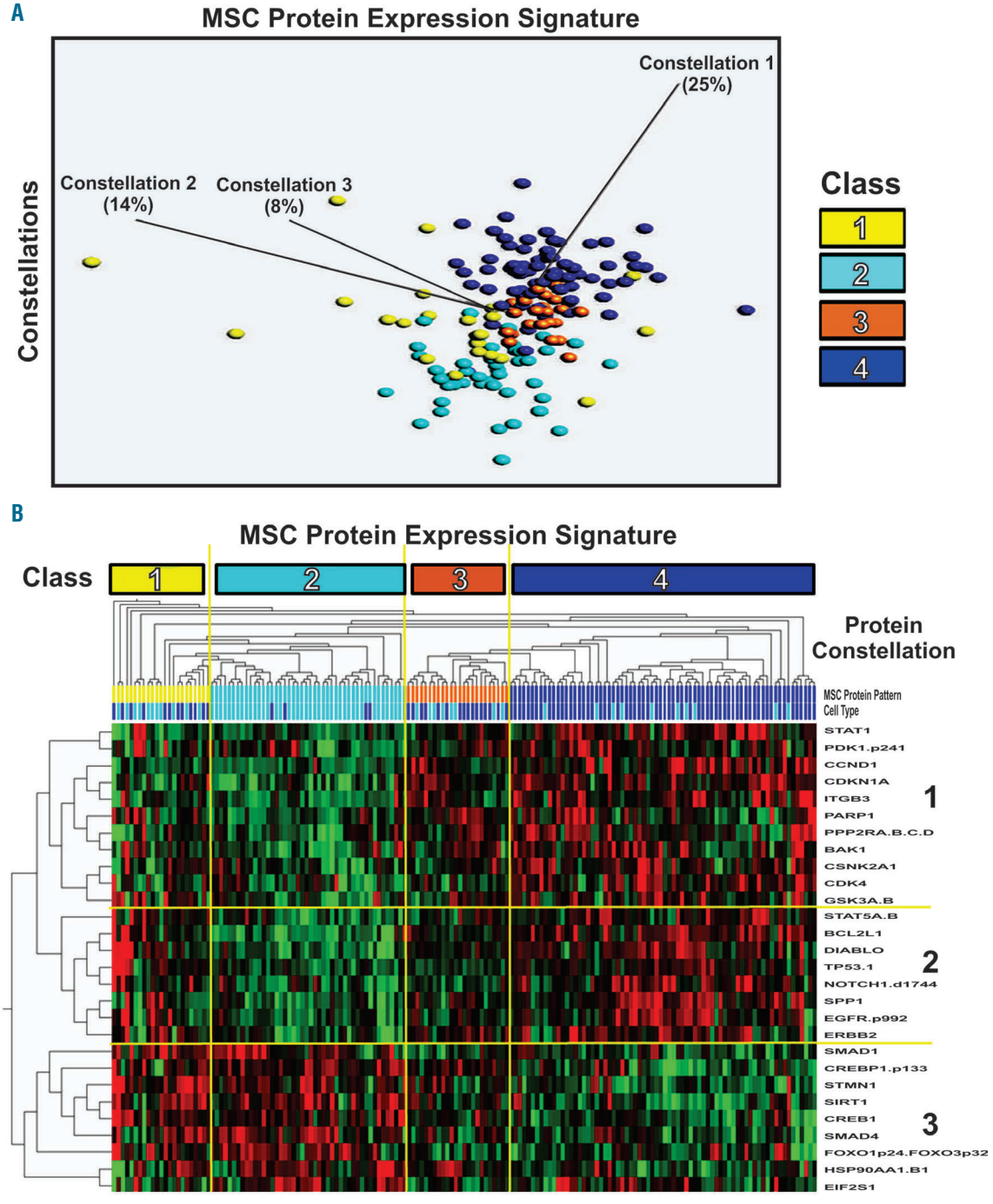

Figure 1. Mesenchymal stromal cell (MSC) protein expression signatures. Protein expression is distinct between normal MSC and acute myeloid leukemia (AML)-MSC. (A) Principal component analysis (PCA) of 151 proteins examined in Class 1 (yellow), Class 2 (light blue), Class 3 (orange) and Class 4 (dark blue). (B) Unbiased hierarchical clustering identifies 3 protein signature groups: Group 1 (11 members), Group 2 (8 members) and Group 3 (9 members) in Class 1 (yellow), Class 2 (light blue), Class 3 (orange) and Class 4 (dark blue) groups, identified in top row as "MSC protein type". MSC derived from normal donor (light blue) or AML patient (dark blue) is shown in the second row marked "cell type".

Table 2. List of proteins with reverse correlation to one or more other proteins in acute myeloid leukemia (AML) mesenchymal stromal cells (MSC) *versus* normal MSC.

Protein	AML-MSC negative normal MSC positive	AML-MSC positive normal MSC negative
AKT1S1	STAT3	
AKT3	BCL2L11, ERG, SFN, SRCp416	
ARC	STAT3 p727	CTNNB1
ATF3	BCL2, KIT, SFN, TP53 p15	
BCL2	ATF3, CTNNB1	
BCL2L11	ATF3, CTNNB1	
BECN1	PSMD9, YWHAE	
BID	GAPDH	BIRC5, GAB2 p452
BIRC5	MS4A1, FN1, SRC p416	BID, EIF2S1 p51, IRS1 p1101, RPS6KB1
CAV1		PTK2
CCNB1	MAPK1.3 p202.p204	
CTNNB1	BCL2, BCL2L11, FOXO1.3 p24.p32, FOXO3, KIT, ITGAL, PSMD9 p10, RPS6KB1 p389, SFN, SRC, SRC p416	ARC, HSP90AA1.B1, MAPK9, PTGS2, YWHAZ
CDK1	CREB1	
CDK4	GAPDH, MAPK1.3 p202.p204, YWHAE	
CREB1	CDK1, JUN p73	DIABLO, PECAM1, SFN
CREB2 p133	MAPK9	TP53
DIABLO	HSPB1	CREB1
EGLN1		FOXO1.3 p24.p32, FOXO3, MS4A1, SRC p416, TCF4, YWHAE
EIF2S1 p51		BIRC5, TGM2
ELK1 p383	SMAD1	
ERBB2 p1248	TCF4	
ERG	AKT3	
FN1	BIRC5	
FOXO1.3/FOXO3 p24.p32	CTNNB1	EGLN1
FOXO3	CTNNB1, IRS1 p1101	EGLN1
GAB2 p452		BID
GAPDH	BID, CDK4, GSK3A.B	
GSK3A.B	GAPDH, STK11	
HDAC3	PSMD9	
HNRNPK		STAT5A.B
HSP90AA1.B1		CTNNB1
HSPB1	DIABLO	
IRS1 p1101	FOXO3	BIRC5
ITGAL	CTNNB1	
JMJD6	STAT3 p727	
JUN p73	CREB1	NRP1
KIT	ATF3, CTNNB1	
LEF1	PTK2	
MAP2K1		XIAP
MAPK1.3 p202.p204	CCNB1, CDK4	SRC p527
MAPK9	CREB2 p133	CTNNB1
MAPK14	STAT1	
MS4A1	BIRC5	EGLN1
NPM1		SMAD1
NR4A1	PECAM1	
NRP1	JUN p73	
PECAM1	NR4A1	CREB1

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PPARG		SMAD1
PRKAA1.2 p172		SRC p527
PSMD9 p10	CTNNB1	
PSMD9	BECN1, HDAC3	
PTGS2		CTNNB1
PTK2	LEF1	CAV1
RPS6KB1 p389	CTNNB1	
RPS6KB1		BIRC5
SFN	AKT3, ATF3, CTNNB1	CREB1, STMN1
SMAD1	ELK1 p383	NPM1, PPARG,
SRC	CTNNB1	
SRC p416	AKT3, BIRC5, CTNNB1, XIAP	EGLN1
SRC p527		PRKAA1.2 p172, MAPK1.3 p202.p204
STAT1	MAPK14, STAT5A.B	
STAT3 p727	AKT1S1	
STAT3 p727	ARC, JMJD6	
STAT5A.B	STAT1	HNRNPK
STK11	GSK3A.B	
STMN1		BCL2L11, SFN
TCF4	ERBB2 p1248	EGLN1
TGM2		EIF2S1 p51
TP53		CREB2 p133
TP53 p15	ATF3	
XIAP	SRC p416	MAP2K1
YWHAE	BECN1, CDK4	EGLN1
YWHAZ		CTNNB1

Based on various combinations of proteins expressed within these three groups, we observed that normal or leukemic MSC samples clustered into four distinct PCs. The first (aqua in Figure 1A; top row of annotations above the heat map in Figure 1B), was comprised predominantly of NL-MSC (42 of 46 members), characterized by lower expression of proteins in constellations 1 and 2, and higher expression of constellation 3 proteins. This was considered to represent the protein expression pattern of NL-MSC. AML cases in this signature are hereafter called Class 2.

A second signature (blue in Figure 1A and B) was comprised predominantly of AML-MSC (62 of 72 members), and was characterized by the opposite protein expression of the NL-MSC signature, with high expression of PC1 and 2 proteins and lower expression of PC3 proteins. This signature is called Class 4. Two groups with expression signatures between that of the Class 2 MSC and Class 4 MSC were also recognized. One signature (yellow in Figure 1A and top row of Figure 1B), comprised of 12 AML-MSC and 11 NL-MSC, mirrored the expression of Class 2 for protein constellation 1, but that of Class 4 for PC2 and 3. AML cases in this group are hereafter referred to as Class 1. The final signature (orange in Figure 1A and top row of Figure 1B), consisting of 16 AML-MSC and 8 NL-MSC, mirrored the expression of Class 4 for PC1 and 3 but had low expression of PC2, similar to Class 2. AML cases from this signature are referred to as Class 3.

Clinical correlation

Among the AML cases, there were very few clinical or laboratory features that showed a statistically significant correlation with the four MSC PCs. Specifically there was no association for continuous variables: age, percent BM or blood blasts, hemoglobin, platelet count, serum lactate dehydrogenase, albumin, creatinine, bilirubin fibrinogen, CD7, CD10, CD13, CD14, CD20, CD33 or CD34, or discrete variables disease status (new *vs.* salvage), race, French-American-British (FAB) Classification, performance status, history of an antecedent hematologic disorder, presence of an FLT3-ITD or presence of an NPM1 mutation (Table 1). However, a few notable associations between MSC protein signature and clinical features were observed. There was a statistical difference in presence of FLT3 D835 mutation (P=0.05). While no Class 1 or Class 2 samples contained FLT3 D835 mutation, 9.4% of Class 4 MSC and 5.6% of Class 3 MSC had the mutation (Table 1). Interestingly, there was a statistical difference in MSC population types between men and women (P=0.03) (Table 1). Men had a higher percentage of Class 4 and Class 3 MSC while women had a higher percentage of Class 1 and Class 2 MSC. At present it is unclear if sex influences MSC biology in the leukemic niche. Sex-specific effects in the microenvironment in leukemia is not unprecedented as integrin-mediated adhesion-triggered activation of GSK3B occurs exclusively in leukemia progenitor cells derived from female AML patients.[35] Total GSK3 and ITGA2 are present in protein constellation 1

and the proteins in this constellation are elevated in Class 3 and Class 4 while they are lower in Class 1 and Class 2. Perhaps the integrin/GSK3 axis is also important in AML-MSC.

Finally, there were statistically significant biases with different cytogenetic groups associating with different MSC protein signatures (P=0.026) (Table 1). Specifically, although few patients had favorable cytogenetics in this dataset, these were found exclusively in the Class 1 and Class 2 signatures. Conversely, patients with unfavorable cytogenetics were not observed in the Class 2 signature, but they were found in relatively equal proportions in the three AML specific signatures (i.e. Classes 1, 3, and 4).

Having determined that leukemic MSC divide AML patients into discrete protein signatures, we next examined whether signature membership affected outcomes. There was no significant difference in median overall survival between the four MSC populations, although the median survival of 105 weeks (wk) in the Class 3 signature was longer than the other three groups at 25, 48 and 58 wk (Figure 2A); this suggested that patients with Class 3 MSC may have a better disease prognosis. Despite the small sample size, relapse rates among the newly diagnosed cases were different, with 4 of 5 Class 1 MSC patients and 13 of 16 Class 4 MSC patients relapsing compared to only 2 of 7 Class 3 MSC cases (P=0.076). Furthermore the median remission duration was different between patients with Class 3 MSC and the three other signatures (101 wk for Class 3 MSC *vs.* 42.2 wk for Class 4 MSC; P=0.05) (Figure 2B). Other comparisons were not made due to the small sample size. These findings suggest that MSC can influence patient survival, although at present the mechanism is unknown.

Effect of age on protein expression in NL-MSC and AML-MSC

As AML is a disease of the elderly, many of the AML-MSC samples were obtained from individuals aged over 60 years (y). Of the 106 AML-MSC samples, 50 samples were from individuals under 60 y. Of the 71 MSC samples from healthy donors, 68 samples were from individuals under 60 y. To assess the impact of age on the differences in protein expression between the AML-MSC and NL-MSC groups, the protein expression of 24 of the differentially-expressed proteins was reassessed in age-matched sets. The three groups were: a) under 30 y (AML n=13; NL n=37); b) 30-49 y (AML n=17; NL n=25); and c) 50 to 59 y (AML n=20; NL n=6). As shown in *Online Supplementary Table S2* and *Online Supplementary Figure S4A*, of the 19 proteins elevated in AML-MSC compared to NL-MSC, only two (i.e. BCL-XL and PPP2R2A/B/C/D) were significantly higher in all age groups. This suggests that increased expression of these 2 proteins is dependent on the disease state and not on age. Six other proteins including p53 and CDKN1A (p21) were also elevated in two of the three age groups suggesting expression of these proteins is also dependent on the disease state rather than on age (*Online Supplementary Table S2*).

Analysis of p53 and CDKN1A (p21) protein expression was performed by western blot analysis on age-matched AML and NL samples (age 40-49 y; n=3 for both groups). Protein expression of both p53 and CDKN1A (p21) was at least 2- to 3-fold higher in AML-MSC compared to the healthy donor samples (*Online Supplementary Figure 4B*). These findings suggest that the disease state dictates expression of at least some of the identified proteins in MSC.

Evidence for dysregulated signaling in AML-MSC

The observed differences in protein expression between AML-MSC and NL-MSC suggest that pathway utilization may be dysregulated or non-canonical in the AML-MSC. To look for evidence of abnormal utilization, we searched for statistically significant (R>0.2; P< 0.0001) protein-protein correlations that were reversed in the direction of correlation in the AML-MSC compared to the pattern in the NL-MSC. A representative analysis of STAT5 expression with the other proteins in NL-MSC revealed that this transcription factor is negatively correlated with hnRPK and positively correlated with STAT1 (*Online Supplementary Figure S5B*). However, in AML-MSC the correlations are reversed (*Online Supplementary Figure S5B*).

A list of all protein correlations that are reversed in AML-MSC compared to NL-MSC is provided in Table 2. Of note, there are significant changes in protein correlations involving β-catenin. As shown in Table 2, BCL2, BCL2L11, FOXO1.3 p24.p32, FOXO3, KIT, ITGAL, PSMD9 p10, RPS6KB1 p389, SFN, SRC, and SRC p416 are negatively correlated with β-catenin in NL-MSC but

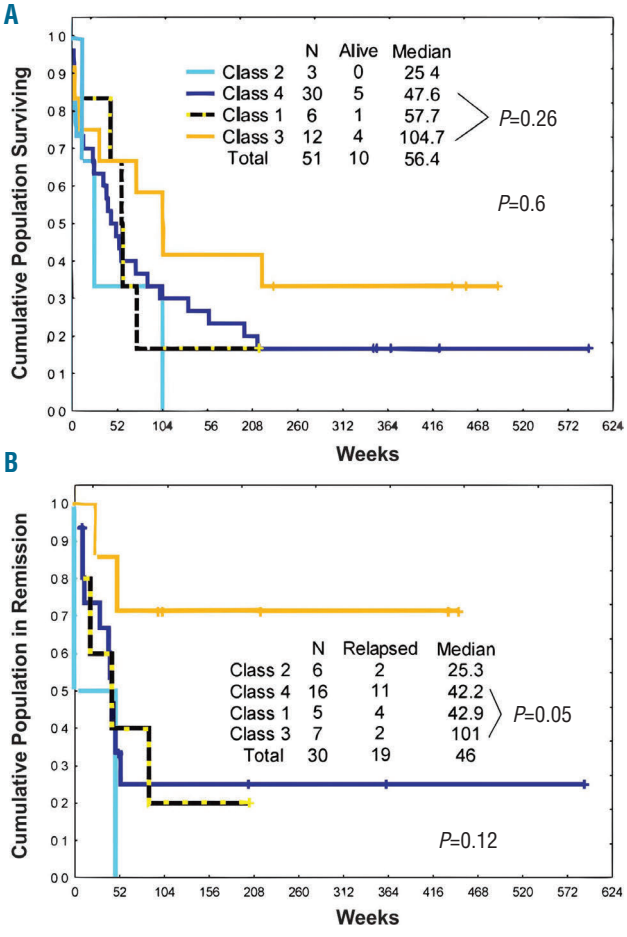

Figure 2. Survival and remission duration differ in patients based on mesenchymal stromal cell (MSC) population. Kaplan-Meir curves showing overall survival (A) and remission duration (B). N: number.

are positively correlated with the protein in AML-MSC. ARC (NOL3), HSP90AA1/B1, MAPK9, PTGS2, and YWHAZ were positively correlated with β-catenin in NL-MSC but are negatively correlated with the protein in AML-MSC. Ingenuity Pathway Analysis (IPA) was performed using software on the proteins identified as differentially correlated with β-catenin in the MSC sets and β-catenin itself (i.e. CTNNB1; BCL2; BCL2L11; FOXO1; FOXO3; KIT; ITGAL; PSMD9; RPS6KB1; SFN; SRC; NOL3; HSP90AA1; HSP90B1; MAPK9; PTGS2; YWHAZ). IPA revealed these proteins were highly associated with PI3K/AKT signaling (top canonical pathway; *P*=7.35E-19) (*Online Supplementary Figure S5*). IPA also identified the top upstream regulator of this set of proteins as p53 (*P*=1.04E-11).

Proteins differentially expressed in AML-MSC share interactomes

To assess the relationship among the proteins identified in the RPPA analysis, protein association network analysis was performed using STRING 10.533 on proteins identified as significantly different in the AML-MSC and NL-MSC (Figure 1B). Blalock *et al.* used a previous version of String software to map the nuclear interactome.[36] In cases where a family of proteins was identified (e.g. PPP2R2 set and HSP90 set), a representative member was included in the analysis. With the exception of PDK-1 all proteins are interconnected at least through one association (Figure 3). This finding suggests that there is an interconnection between the various proteins that are distinctly expressed between the NL-MSC and AML-MSC groups.

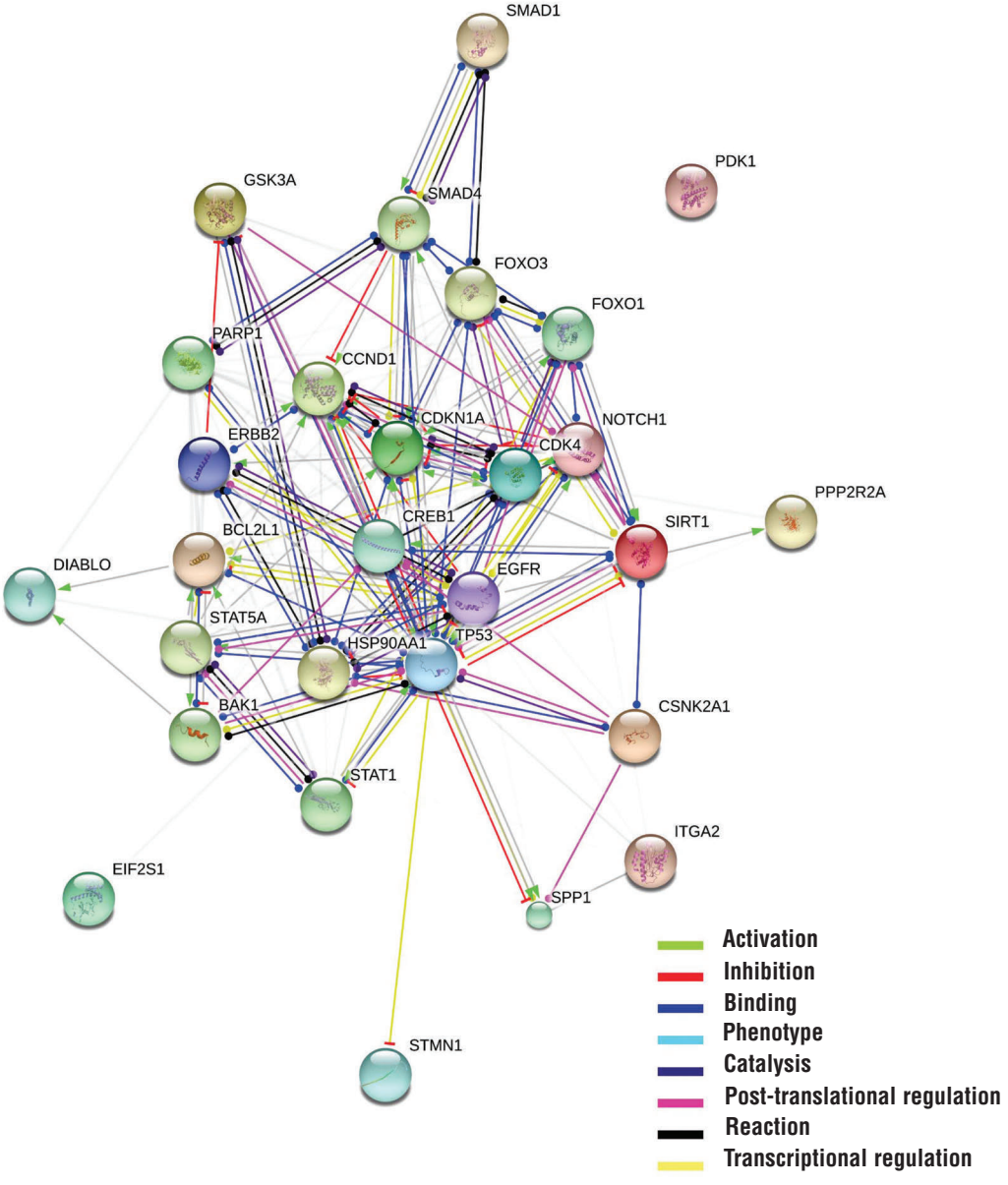

Figure 3. Proteins differentially expressed in acute myeloid leukemia (AML) and normal mesenchymal stromal cell (MSC) are highly interactive. (A) String analysis was performed by String 10.0 using interactions based on "action"; available from: *http://string-db.org*. (B) Model of involvement of Group 1 and 2 proteins in AKT signaling. Red: proteins are members of Group 1 or 2. Yellow; proteins are non-members but possible links.

Pathway analysis suggests NL-MSC have prominent adipogenic signaling while all AML-MSC populations have prominent PI3K/AKT signaling

Pathway analysis was performed using IPA. Two separate data sets were created: one with proteins from PC3 (elevated in normal MSC) and one with proteins from PC1 and 2 (elevated in AML-MSC). Proteins in group 3 are associated with adipogenesis (ninth top canonical pathway; *P*=2.19E-05) (*Online Supplemenly Figure S6*). PC3 proteins are elevated in MSC that were presumably normal but reduced in AML-MSC suggesting differences in differentiation potential of MSC between NL-MSC and AML-MSC. Of the 7 proteins identified, SIRT1, FOXO1, and SMAD1 each can activate PPARβ which is a critical regulator of adipocyte differentiation.[37-39] PC3 also displayed the strongest association with AMPK signaling (*P*=6.84E-07). The top upstream regulator identified in the set of PC3 proteins was PDGFB (*P*=1.01E-06). PI3K/AKT pathway was highly associated with PC1 and 2 proteins, which are elevated in AML-MSC (top canonical pathways; *P*=7.16E-17) (*Online Supplementary Figure S7*). AKT was identified as one of the top three upstream regulators (*P*=1.22E-14), suggesting that signaling mediated by this survival kinase is prominent in AML-MSC.

AML-MSC are senescent compared to NL-MSC

The p21 protein appears to be critical for senescence in myelodysplastic syndrome (MDS)-MSC22 and AML-MSC similar to MDS-MSC have elevated p21 (Figure 1B). This finding suggests that AML-MSC might also be more senescent than NL-MSC, as was the case in MDS-MSC.[25] Senescence was observed in normal donor MSC- and AML-derived MSC using β-galactosidase staining. AML-MSC were more senescent than MSC derived from healthy donors in this representative example (Figure 4A). To account for age effects on senescence, MSC were taken from donors under 58 y. Average age of the AML patients (n=4) was 52 y and the average age of normal donors (n=5) was 47 y. Also, MSC of similar cell passage (passage 2 or 3) were used, so effects of cell passage were unlikely. As shown in Figure 4B, β-galactosidase activity was significantly higher (almost 2-fold) in AML-MSC compared to

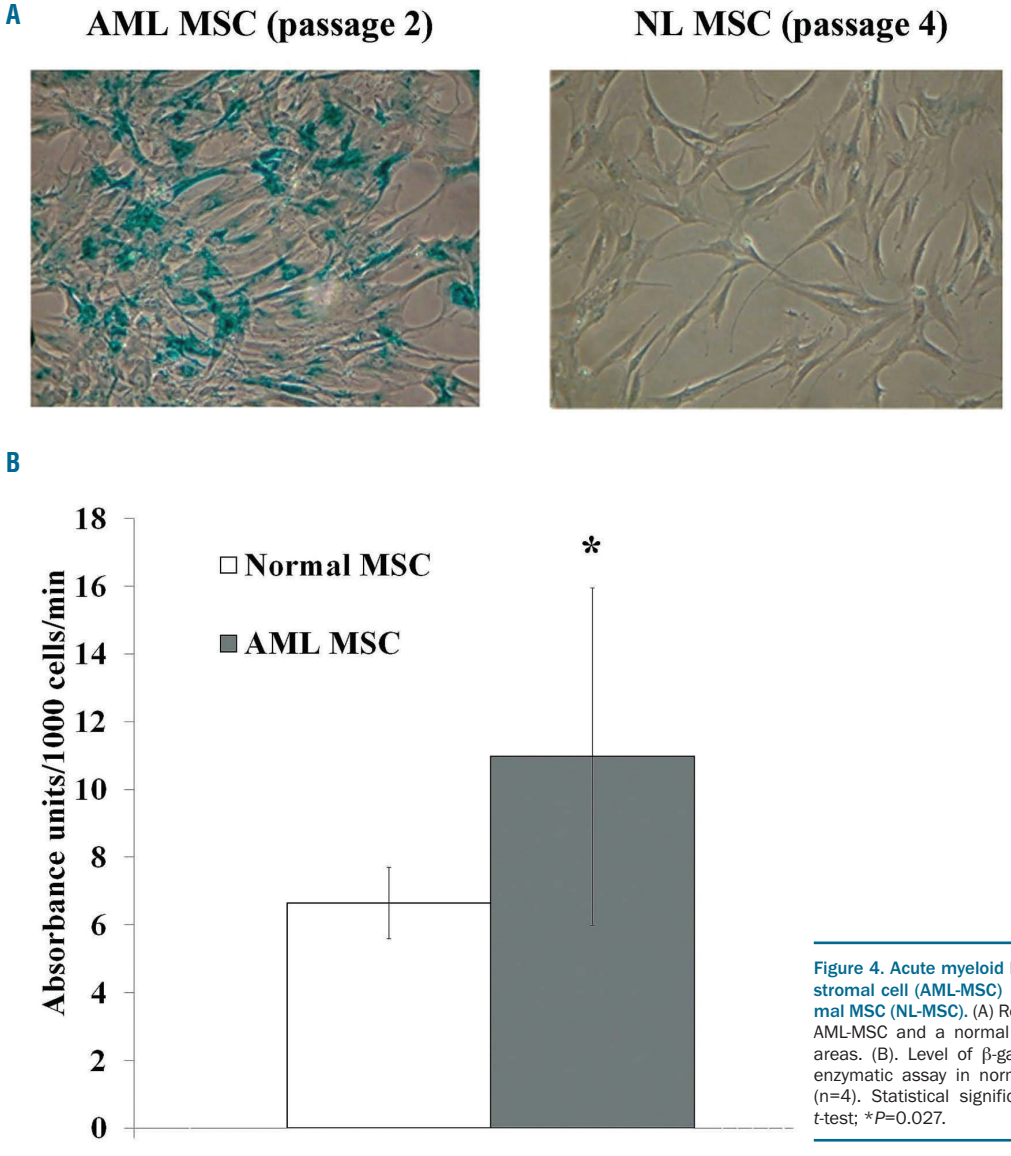

Figure 4. Acute myeloid leukemia (AML) mesenchymal stromal cell (AML-MSC) are more senescent than normal MSC (NL-MSC). (A) Representative microscopy of an AML-MSC and a normal MSC with two different slide areas. (B). Level of β-galactosidase was assessed by enzymatic assay in normal MSC (n=5) and AML-MSC (n=4). Statistical significance determined by Student *t*-test; **P*=0.027.

NL-MSC (P=0.032). These findings suggest that AML-MSC tend toward senescence.

Therapy alters proteins expression in AML-MSC

Protein expression in AML-MSC might change between diagnosis and relapse, perhaps as a result of relapse, or in response to acquired changes in AML blasts. To determine if AML-MSC protein expression was changing between diagnosis and relapse we compared expression between the samples obtained at first diagnosis (n=53) to those of AML-MSC collected from primary refractory or relapsed patients (n=54). Nine proteins are differentially expressed between the two MSC groups (Figure 5). Phosphorylated β-catenin, phosphorylated RPS6, and galectin-3 are expressed at higher levels in MSC in the salvage set. SMAD6, TCF4, LYN, integrin-β3, phosphorylated EIF4BP1, and phosphorylated ELK1 are higher in MSC at first diagnosis compared to MSC from salvage AML patients. IPA reveals that, for canonical pathways, a set associated with osteoblast differentiation was found to be the pathway most associated with the proteins differentially expressed at diagnosis compared to salvage (*Online Supplementary Figure S8*).

Discussion

This study presents the first systematic study of protein expression differences between NL-MSC and AML-MSC. There were several notable observations. There were clear differences between the protein expression of MSC (whether from healthy donor or AML patient) and AML blast cells. This result was not surprising as one would predict different proteins would be prominent in mesenchymal cells and cells of hematopoietic/myeloid lineage. The major observation of this research was the discovery that AML-MSC have significantly different protein expression patterns compared to normal MSC, with 28 of 151 analyzed proteins being highly significantly different (FDR<0.006). These changes assumed four signatures in AML, 81% of which were very different from that of normal MSC, while 6% had an identical signature to NL-MSC, and another 13% were more like the normal signature than the leukemia patterns. Signature membership showed an association with cytogenetics, with 'favorable' cytogenetics being limited to the more NL-MSC-like pattern and 'unfavorable' cytogenetics not occurring in AML with an NL-MSC-like pattern. There was a difference in the distribution of the MSC population between men and women. The significance of these differences is not clear, but women tended to have higher percentages of Class 1 and Class 2 MSC compared to men. In leukemia progenitor cells, GSK3B is activated *via* an integrin-mediated mechanism in response to adhesion to a stromal cell exclusively in women patients.[35] As ITGA2 and GSK3 are members of a protein constellation (i.e. constellation 1) that is differentially expressed in Class 1 and Class 2 (lower levels) compared to Class 3 and Class 4 (higher levels), it is tempting to speculate that integrin/GSK3 axis may contribute to sex-specific effects in MSC.

Furthermore, these signatures influence outcomes including response rates, remission duration and, perhaps, survival. Patients with Class 3 MSC fare much better than patients with Class 4 MSC as demonstrated by significantly longer remission duration and a trend toward longer OS. Changes in protein expression were often characterized by protein-protein correlations that were reversed from those seen in normal MSC, providing insight into the nature of this dysregulation and potentially providing therapeutic targets. In NL-MSC, the signature proteins were associated with adipocyte differentiation. That normal MSC, but not AML-MSC, possess protein pathways impor-

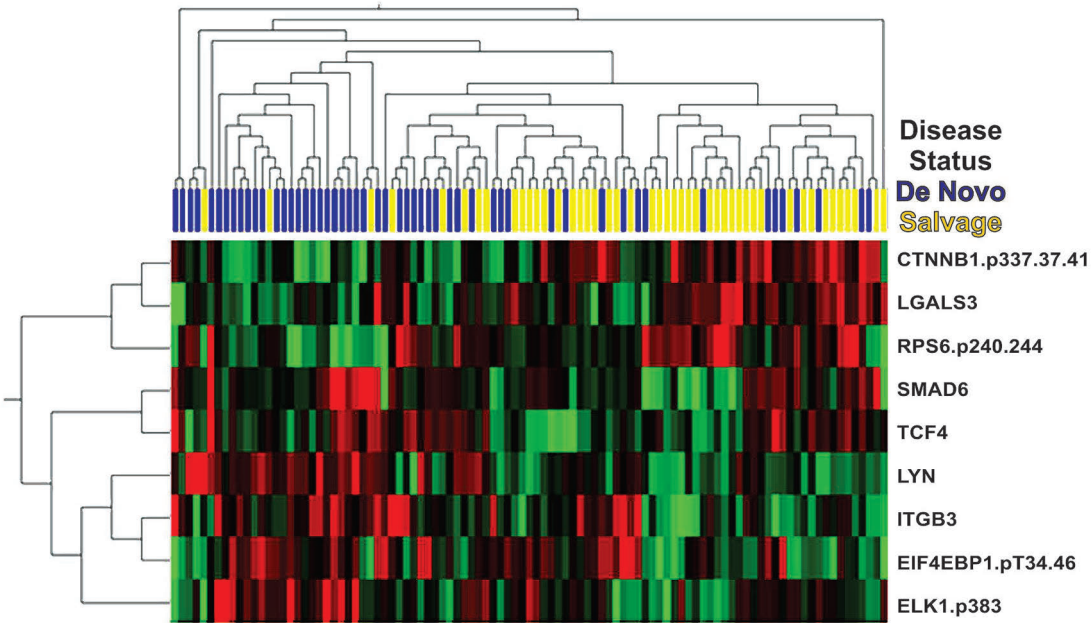

Figure 5. A distinct set of proteins is associated with acute myeloid leukemia (AML) patient salvage status. (A) Reverse phase protein arrays (RPPA) reveals protein expression in AML mesenchymal stromal cells (MSC) differs between diagnosis and the salvage setting for 9 proteins (P=0.05; false discovery rate=0.68).

tant in adipogenesis is consistent with other studies from our group and another group that found AML-MSC are primed toward osteoblastic differentiation and not adipocytic differentiation.[9,40,41] We reported that AML-MSC express higher levels of osteogenic markers, including Tissue Non-specific Alkaline Phosphatase (TNAP), RUNX2, Osterix, and Ostepontin, compared to MSC from age-matched heathy donors.[40] In addition, in that study we found that AML-MSC readily differentiate along the osteogenic lineage pathway but are unable to differentiate into adipocytes. This differentiation potential of the MSC may be influenced by the leukemia cells themselves, as exposure of healthy donor MSC to AML cell lines such as OCI-AML3 induces gene expression of *RUNX2, TNAP,* and other osteogenic genes, and induces osteogenic differentiation of the stromal cells.[40] The protein networks prevalent in NL-MSC that we identify here are consistent with signaling that skews toward adipocytic differentiation in the normal cells. Diaz de la Guardia *et al.* found that MSC from a high-risk AML group failed to differentiate into adipcocytes.[41] The IPA data on canonical pathways in the AML-MSC compared at first diagnosis with refractory and relapse samples suggests the importance of MSC of the osteoblastic lineage in the AML niche, as many of these proteins are associated with osteoblast survival and differentiation.

PI3K/AKT is very prevalent in group 1 proteins which are associated with Class 4 MSC by IPA. We have previously reported that leukemia cells in co-culture with MCS induce activation of AKT and other survival kinases.[42] It is plausible that the observed activation of AKT signaling in AML-MSC is due to the presence of leukemia cells in the niche or at least that the malignant cells may contribute to activation. In MSC, AKT has been implicated in positive regulation of Cyclin D1 (CCND1) and CDK4.[43,44] The presence of the PP2A B55 α subunit in group 1 suggests that either the protein phosphatase is not active against AKT in those cells or that AKT is less active in the AML-Like MSC compared to Normal-Like MSC. Despite its tumor suppressor role in suppression of AKT signaling, the PP2A subunit does support β-catenin expression by dephosphorylating serine and threonine residues that are required for destruction of the transcription factor (e.g. serine 33, threonine 41).[45-47] It is interesting that when expression of the 151 proteins surveyed by RPPA are correlated in the normal MSC and in the AML-MSC, β-catenin exhibits the greatest difference in correlation pattern between normal MSC and AML-MSC compared to the other proteins. It is plausible that the PP2A B55 α subunit may be a factor in this phenomenon, though further investigation will be required to verify this potential mechanism. It is interesting to note that suppression of PP2A (albeit *via* the catalytic core subunit Cα) in MSC cell line or pre-adipocyte cells results in differentiation to adipocytes *via* a mechanism involving loss of β-catenin.[48] As pathway analysis of proteins associated with normal MSC (i.e. Constellation 3) pathways identifies adipogenesis as a key pathway, it will be important to determine if AML-MSC would be less likely to skew toward adipocyte differentiation because of a PP2A B55 α subunit/β-catenin axis.

BCL-XL is necessary for MSC survival during differentiation.[27] AML-MSC expressed higher BCL-X_L and Cyclin D1 protein. These findings may be attributed to STAT5 as this transcription factor is a regulator of both BCL-X_L and Cyclin D1.[49] We found by qRT-PCR that gene expression of both BCL-X_L and Cyclin D1 was higher in AML-MSC (n=9) compared to normal MSC (n=10) (*Online Supplementary Figure S9*). These findings suggest that elevation of BCL-X_L and Cyclin D1 protein in AML-MSC can be attributed at least in part to a transcriptional mechanism possibly involving STAT5.

Two prominent proteins identified as elevated in the AML-MSC group are p21 and p53 (Figure 1B). Elevated expression of p21 and increased senescence of MSC is consistent with the study on MDS-MSC that showed a role for p21 in IL-6 and TGF-β production.[25] However, a recent study from Desbourdes *et al*. found p21 and p53 levels were similar between AML-MSC and healthy donor-derived MSC.[50] The reason for the difference between our results and their results is not clear. It should be noted that the Desbourdes *et al*. study used less than 5 samples each of AML-derived MSC and healthy donor-derived MSC to determine p21 and p53 levels, so perhaps Class 1 or Class 2 MSC (which would have lower levels of p21 and p53) are over-represented in their samples.[50] In addition, the average age of the AML patients in the Desbourdes *et al*. study was 49 y while the average age of the healthy donors was near 60 y, so perhaps the p21 and p53 levels in the healthy donor MSC are skewed higher as the donors are older than the patients. For p21 and p53 expression, an age match comparison of p21 and p53 in the AML-MSC and NL-MSC shows levels of these proteins are higher in the AML-MSC in at least 2 ages for each (*Online Supplementary Table S2*).

In conclusion, proteomic analysis identified a distinct set of proteins that distinguish normal MSC from AML-MSC. Our RPPA studies identified four major signatures of MSC in AML patients that may impact their function in the tumor microenvironment.

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