ActivinA: a new leukemia-promoting factor conferring migratory advantage to B-cell precursor-acute lymphoblastic leukemic cells

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 doi:10.3324/haematol.2018.188664

Received: January 24, 2018.

Accepted: September 21, 2018.

Pre-published: September 27, 2018.

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Supplementary methods

Patient and healthy donors (HDs) samples

BM plasma samples were collected from 125 BCP-ALL patients (median:4.6; range:1-17 years-old) at disease diagnosis and from 56 healthy donors (HDs) (median:17; range:5-51 years-old). Primary BCP-ALL cells were isolated at disease diagnosis from 22 BM aspirates (>85% blast infiltrate) by Ficoll (GE Healthcare, Uppsala, Sweden) gradient separation and cryopreserved in liquid phase nitrogen until usage.

Informed consent to participate to the study was obtained for all BCP-ALL patients and HDs. All BCP-ALL patients were enrolled in the AIEOP-BFM ALL 2009 protocol (EudraCT-2007-004270-43) and their samples collected at the Pediatric Department of Fondazione MBBM/San Gerardo Hospital (Monza, Italy) or at Bambino Gesù Hospital (Rome, Italy). Enrolled HDs were stem cell donors whose BM was collected at the Pediatric Department of Fondazione MBBM/San Gerardo Hospital (Monza, Italy) for transplant purposes.

Culture of BCP-ALL cell lines

The leukemic cell lines 697, NALM-6, RS4;11, SUP-B15 and REH (ATCC, Milan, Italy) were cultured in Advanced RPMI 1640 (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (GE Healthcare), penicillin (100 U/mL), streptomycin (100 µg/mL) and L-glutamine (2 mM) (Euroclone, Milan, Italy). Cell line biological identity was analyzed by cell surface phenotyping (flow cytometry, FACS Canto II, BD Bioscience, San Jose, CA, USA) and detection of cell-specific translocations.

Isolation of BM-MSCs

For BM-MSC isolation, BM-MNCs were seeded in low glucose Dulbecco's Modified Eagle Medium (Lonza, Milan, IT), 10% heat-inactivated FBS, penicillin (100 U/mL), streptomycin (100 µg/mL) and L-glutamine (2 mM). After 48 hours, non-adherent cells were removed from the culture by washing with PBS without calcium and magnesium (Euroclone). When 70-80% confluence was achieved, adherent spindle-shaped cells were harvested using 0.05% trypsin-EDTA (Euroclone) and used for further expansion. After three culture passages, BM-MSCs were analyzed by flow cytometry. All the isolated BM-MSCs lines resulted positive for CD105, CD73, CD90, CD54, MHC-I expression, while they resulted negative for CD45, CD34, CD14 and MHC-II expression (anti-human CD14, CD90 and CD105: eBioscience; anti-human CD45, CD54, CD73, MHC-I and MHC-II: BD Pharmingen; anti-human CD34: BD Biosciences). To evaluate their osteogenic and adipogenic differentiation ability, BM-MSCs were stimulated for 14-21 days with specific

differentiation inductive media. Adipogenic inductive medium consisted of DMEM-High glucose (Euroclone) supplemented with 10% FBS, penicillin (100 U/mL), streptomycin (100 µg/mL) and L-glutamine (2 mM), dexamethasone (1 µM), indomethacin (1 µM), 3-isobutyl-1-methylxantine (IBMX) (500 µM) and human recombinant insulin (10 µg/ml) (all from SigmaAldrich, St Louis, MO, USA). Lipid droplets were stained with Oil Red O (SigmaAldrich). Osteogenic inductive medium consisted of DMEM-Low glucose, 10% FBS, 2-phosphate-ascorbic acid (50 µM), β-glycerol phosphate (10 mM) and dexamethasone (100 nM) (all from SigmaAldrich). The presence of calcium deposits was detected by Alizarin Red staining (SigmaAldrich).

AGE AT DIAGNOSIS	SEX	DIAGNOSIS	% OF BM INFILTRATE	TRANSLOCATIONS	MRD RISK
3	M	B-II	90	NEG*	HR (SER)
3	M	B-II	98	t(12;21)	IR
4	M	B-II	90	NEG*	SR
4	F	B-II	77	NEG*	HR (SER)
4	M	B-II	86	t(12;21)	IR
6	M	B-II	80	NEG*	HR (SER)
6	F	B-II	95	NEG*	IR
7	M	B-II	59	t(12;21)	IR
7	F	B-II	98	t(12;21)	IR
8	F	B-II	88	t(12;21)	SR
9	F	B-II	94	NEG*	HR
9	M	B-II	78	NEG*	HR
10	M	B-II	N.A.	t(9:22) p210	HR
15	M	B-II	89	NEG*	IR
18	M	B-II	86	NEG*	IR

Supplementary Table 1. Clinical and molecular features of BCP-ALL patients enrolled for BM-MSC isolation. MRD risk was defined on the basis of residual leukemic cells in the BM at day +15, +33, +78 after treatment beginning (HR: HIGH RISK, IR: INTERMEDIATE RISK, SR: STANDARD RISK, SER: SLOW EARLY RESPONDER). *NEG: negative for t(4:11), t(9;22) p190, t(12;21), t(1;19).

CB- and BM-CD34$^+$ cell isolation

Cord blood (CB) units from healthy neonates were obtained from San Gerardo Hospital, Monza (BM-Niche Protocol, approved by the Host Institution). Human BM samples were obtained from healthy BM donors at the Pediatric Department of Fondazione MBBM/San Gerardo Hospital

(Monza, Italy; AIEOP-BFM ALL 2009 Protocol). Mononuclear cells were isolated by Ficoll density gradient centrifugation and $CD34^+$ cells were purified using immunomagnetic CD34 microbeads (CD34 MicroBead Kit, Miltenyi Biotec, Cambridge, MA). The purity was assessed by flow cytometry and was consistently >95%.

Quantitative RT-PCR

Total RNA was isolated from the 697 cell line or primary BCP-ALL cells treated or not with ActivinA for 6 hours and 24 hours using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and used for cDNA preparation (Superscript reverse transcriptase, Invitrogen). qRT-PCR was performed using LightCycler® 480 (Roche, Basel, Switzerland). Primers and probes, synthesized with Universal Probe Library (UPL, Roche) software, are listed in Supplementary Table 2. Gene expression levels of target genes were normalized on GAPDH levels.

TARGET	PRIMER	SEQUENCE
ALK2	Forward	acactccccacgggaaac
	Reverse	aaagaagagaagcacaggcaat
ALK4	Forward	gaagtgcagcccctctca
	Reverse	cgtctccactggcagtctc
ACVR2A	Forward	aaagcccagttgcttaacga
	Reverse	tgccatgactgtttgtcctg
ACVR2B	Forward	gcataagctgggttttctcct
	Reverse	cctgagcaactcatgcaaag
ATP2B2	Forward	tgcaggattggtggtgatt
	Reverse	tccctggaactggcatctac
ATP2B4	Forward	ccttgtctttgcgggtga
	Reverse	ggctgggtggtgaatgtaga
ARHGAP25	Forward	tggcccgaagctctgtag
	Reverse	tggttgtatcagagtcgcttgt
CORO1A	Forward	agtttgtggccctgatctgt
	Reverse	cattcttgtccacacgtcca
DOCK4	Forward	agcccgatgagaccatctt
	Reverse	gctctctggaatgggagtca
GAPDH	Forward	agccacatcgctcagacac
	Reverse	gcccaatacgaccaaatcc
LCK	Forward	agtcagatgtgtggtcttttgg
	Reverse	cctccgggttggtcatc
PTPRC	Forward	ttcatgcagctagcaagtgg
	Reverse	gccgtgtccctaagaaacag
VAV3	Forward	ccttagatacaactctgcagtttcc
	Reverse	gcccagcacttttggactta

Supplementary Table 2. Primer sequences for quantitative real-time PCR.

Gene expression profile (GEP) analysis

RNA from four independent experiments was extracted using TRIzol reagent (Invitrogen) and quality and purity were assessed on the Agilent Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany). RNA concentration was determined using NanoDrop ND-2000 Spectrophotometer (Thermo Scientific, Waltham, USA). *In vitro* transcription, hybridization and biotin labeling were performed according to 3'IVT Plus Reagent Kit (Affymetrix, Santa Clara, CA, USA). All data analyses were performed in R (http://www.R-project.org/ version 3.0.2) using Bioconductor and R packages. Probe level signals were converted to expression values using the robust multi-array averaging (RMA) algorithm[1].

Supervised analyses were performed using shrinkage test[2] and multiplicity corrections were used to control FDR (false discovery rate); probes with local FDR lower than 0.05 were considered significant. Gene ontology (GO) and KEGG pathway analyses were performed using Metascape (http://metascape.org)[3] on selected Affymetrix IDs. Enriched terms, hypergeometric p-value and enrichment factors were calculated and used for filtering from the Software. Remaining significant terms were then hierarchically clustered in a tree based on kappa-statistical similarities among their gene membership. Then 0.3 kappa score was applied as the threshold to cast the tree into the term clusters. In the dendogram the 20 best p-value were used in the representation. Heatmap cells were colored according to their p-value. Grey cells indicate the lack of enrichment for that term in the corresponding gene list.

Time-lapse microscopy

Leukemic cells were seeded (3×10^3 cells per well) in Advanced RPMI 1640 1% FBS in a 8-well chamber slide (Ibidi, Martinsried, Germany), previously coated with a 1% Gelatin B solution in PBS (SigmaAldrich). After overnight incubation to promote adherence, cells were stained with Propidium Iodide (PI) to discrimate live cells and stimulated or not with ActivinA. For cell tack recording, chamber slides were mounted on a heated stage within a temperature-controlled chamber maintained at 37°C and constant CO_2 concentrations (5%). Images were acquired over 24 hours. Frame-by-frame displacements and velocities of randomly selected leukemic cell movements were calculated by tracking individual cells using ImageJ software (NIH) on manual tracking mode.

Chemotaxis assays

After ActivinA pretreatment, leukemic and healthy $CD34^+$ cells were resuspended in Advanced RPMI 1640 1% FBS and allowed to migrate through Transwell inserts (5 μm pore size, PC membrane) for 4 hours (BCP-ALL cell lines) or 1 hour (BCP-ALL primary blasts and healthy

$CD34^+$ cells). Advanced RPMI 1640 1% FBS containing or not CXCL12 (100 ng/mL, if not otherwise specified) (Peptrotech, Rocky Hill, NJ) was added to the lower chambers.

For ActivinA receptors blockade experiments, BCP-ALL cell lines (697 and NALM-6) and primary blasts were pretreated for 1 hour with either SB431542 (SigmaAldrich) or vehicle (DMSO, SigmaAldrich) before the addition, or not, of ActivinA (50 or 100 ng/mL, respectively). After 24 hours cells were collected, washed and resuspended at a concentration of 0.5×10^6/100uL in Advanced RPMI 1640 1% FBS added of SB431542 or DMSO. After 1 hour, ActivinA was added or not and cells were allowed to migrate through Transwell inserts (5 μm pore size, PC membrane) for 4 hours (BCP-ALL cell lines) or 1 hour (BCP-ALL primary blasts). Advanced RPMI 1640 1% FBS containing or not CXCL12 (100 ng/mL) (Peptrotech) was added to the lower chambers. The percentage of migrated cells in response to CXCL12 was calculated over input cells for the following experimental conditions: ActivinA-treated cells + DMSO (A); ActivinA-treated cells + SB431542 (B); unstimulated cells + DMSO (C). The percentage of inhibition was calculated as (A-B)/(A-C)*100.

Migrated cells were counted by flow cytometer on a FACS Canto II cytometer (BD Biosciences) after adding a known number of fluorescent reference beads (BD Trucount tubes, BD Bioscience). Technical duplicates were performed for each condition. Input cells and cells harvested from each well were counted twice, for 60 seconds. The percentage of migrated cells was determined by dividing the number of cells in the lower chamber by the total input of cells added to the upper chamber.

Invasion assays

Membrane filters (Costar Transwell® Permable Supports, Corning Inc., pore size 8 μm) were coated with 50 μL of Matrigel (1mg/mL) (Corning) that was allowed to form a gel layer for 1 hour at 37 °C. BCP-ALL cell lines and primary blasts treated or not with ActivinA (50 or 100 ng/mL, respectively) were loaded on the upper chamber (5×10^5 cells/well in 100 μL Advanced RPMI 1640 1% FBS). The medium containing or not CXCL12 (100 ng/mL) was added to the lower chamber. The percentage of cells migrated after 24 hours through the Matrigel barrier into the lower chamber was quantified as described for chemotaxis assay.

Activin Receptor analyses

The expression of Activin Receptors ALK4, ACVR2A and ACVR2B was evaluated by flow cytometry, using the following anti-human antibodies: anti-Activin RIB/ALK-4 Alexa Fluor® 488-conjugated mAb (R&D Systems); anti-human Activin RIIA APC-conjugated mAb (R&D Systems);

primary anti-human Activin Receptor Type IIB mAb used in combination with secondary goat anti-mouse IgG H&L DyLight® 488-conjugated mAb (Abcam, Cambridge, UK). To ensure the specificity of the staining, Fc Receptor Binding Inhibitor Polyclonal Antibody (eBioscience, CA, USA) was used. Mouse IgG APC-conjugated antibody (R&D Systems), mouse IgG FITC-conjugated antibody (BD Biosciences) were used as isotype controls for directly stained antibodies. Concerning ACVR2B indirect staining, the secondary antibody mouse IgG H&L DyLight® 488-conjugated mAb (Abcam) was used alone as negative control. Cells were analyzed with a FACS Canto II cytometer (BD Biosciences) and data analyzed by FlowJo software (Tree Star, Inc. Ashland, OR, USA).

ALK2 expression was evaluated by Western Blot analyses on BCP-ALL cell extracts. For this purpose, cells were washed with ice cold PBS and lysed for 30 minutes on ice. Cell extracts were prepared in lysis buffer containing 1% NP-40 (SigmaAldrich), 0.5% Na-Deoxyxholate (SigmaAldrich), 350 mM NaCl (SigmaAldrich), 0.1% SDS (SigmaAldrich), 1% Protease Inhibitor (SigmaAldrich) and 0.25 mM PMSF (SigmaAldrich) in PBS. Cell debris were removed by centrifugation at 21'000 × g at 4 °C for 5 minutes and protein concentration measured using Pierce BCA Protein Assay Kit (Thermo Scientific). Samples were then separated on Any kD™ Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad, CA, USA). Gels were blotted onto Immun-Blot® PVDF Membranes (Bio-Rad), blocked with 10% dried milk in Tris-buffered saline with 0.1% Tween 20 (SigmaAldrich) (TBS-T) and incubated with primary antibodies. Primary antibodies used were rabbit monoclonal anti-human Activin Receptor Type IA antibody (Abcam) and mouse monoclonal anti-β-Actin antibody (SigmaAldrich). Blots were washed in TBS-T before incubation with horseradish peroxidase conjugated secondary antibodies, goat anti-rabbit IgG (Invitrogen) and rabbit anti-mouse IgG (SigmaAldrich). The blots were washed thoroughly with TBS-T before bands detection using LiteAblot Extend (Euroclone) as luminescence substrate. Image detection was performed with Alliance LD2-77WL system (Uvitec, Cambridge) and image quantification with ImageJ software (NIH, USA). In detail, the ALK2 57 kDa band was quantified by densitometric analysis and normalized to the constitutive protein β-actin.

Concerning $CD34^+$ cells, Activin receptor expression was evaluayed by qRT-PCR. In addition, Activin receptor modulation was evaluated on 697 cells pretreated or not with ActivinA (50 ng/mL) for 6, 24 or 48 hours by qRT-PCR. Primers for qRT-PCR are listed in Supplementary Table 2.

CXCR4 and CXCR7 staining

PE-conjugated anti-human CXCR4 mAb (BioLegend, San Diego, CA) or PE-conjugated anti-human CXCR7 mAb (BioLegend) were used. For intracellular detection, the cells were firstly permeabilized with Cytofix/Cytoperm (BD Biosciences) and then incubated with the above-mentioned antibodies. Cells were analyzed with a FACS Canto II cytometer (BD Biosciences) and data analyzed by FlowJo software (Tree Star, Inc. Ashland, OR, USA)

Filamentous (F)-actin polymerization assay

697 cells were starved overnight in RPMI 1640 1% FBS and pretreated or not with ActivinA (50 ng/mL) for additional 24 hours. Cells were then harvested and resuspended in culture medium at 37 °C with or without CXCL12 (100 ng/mL) for 15, 30, 60, 120, 180 seconds. To block stimulation, cells were rapidly put on ice and washed with ice-cold PBS. Cells were then permeabilized using Cytofix/Cytoperm and stained with AlexaFluor 647-labeled phalloidin (Invitrogen) following manifacturer's instructions. Cells were analyzed with a FACS Canto II cytometer and MFI was determined for each sample. MFI percentage change was calculated for each time point over the basal value (unstimulated cells).

Calcium mobilization

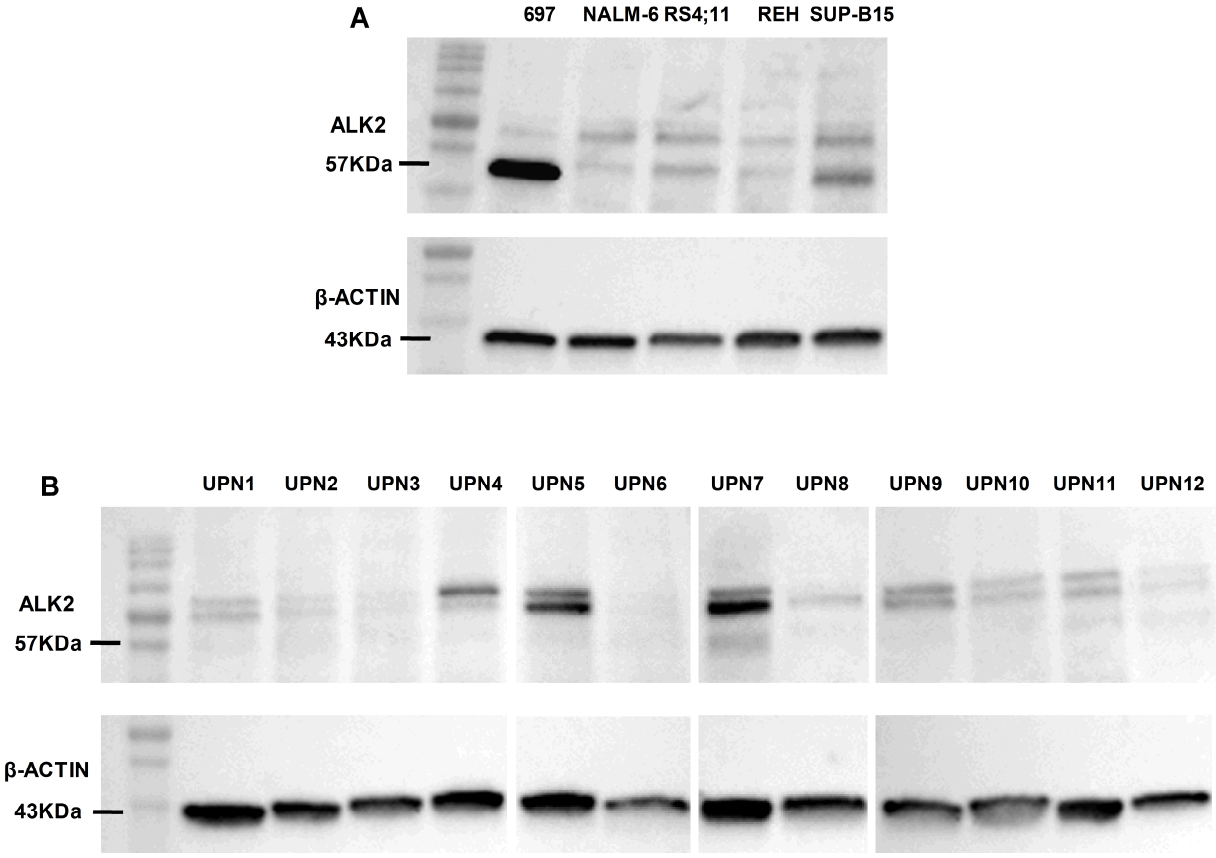
To evaluate intracellular calcium mobilization 697 cells, NALM-6 cells, BCP-ALL primary blasts and CB-$CD34^+$ cells were plated in 96-well plates in Advanced RPMI 1% FBS for 24 hours with or without ActivinA (50 or 100 ng/mL). Cells were then loaded with the cell-permeant Fluo-4NW dye, according to manifacturer's instructions. Analyses were performed by flow cytometry on a FACS Canto II cytometer. After a 30-second baseline recording, sample aspiration was briefly paused and CXCL12 (100 ng/mL) was quickly added. The Ca^{2+} response was measured as MFI of the cells as a function of time. Data analysis was performed using FlowJo "kinetics" tool.

B-cell acute lymphoblastic leukemia xenograft model

7- to 9-week-old NOD-SCID-$\gamma chain^{-/-}$ (NSG) female mice were obtained from Charles River Laboratories-Italy (Calco, Italy) and housed in dedicated rooms with pathogen-free, individually ventilated cages (IVC, Tecniplast spa, Varese, Italy) in the animal facility of the University of Milano-Bicocca (Monza, Italy). All the procedures involving animals handling and care were in accordance with protocols approved by Milano-Bicocca University, in compliance with national and international law and policies. This study was approved by the Italian Ministry of Health (approval n.64/2014, issued on the 4th December 2016).

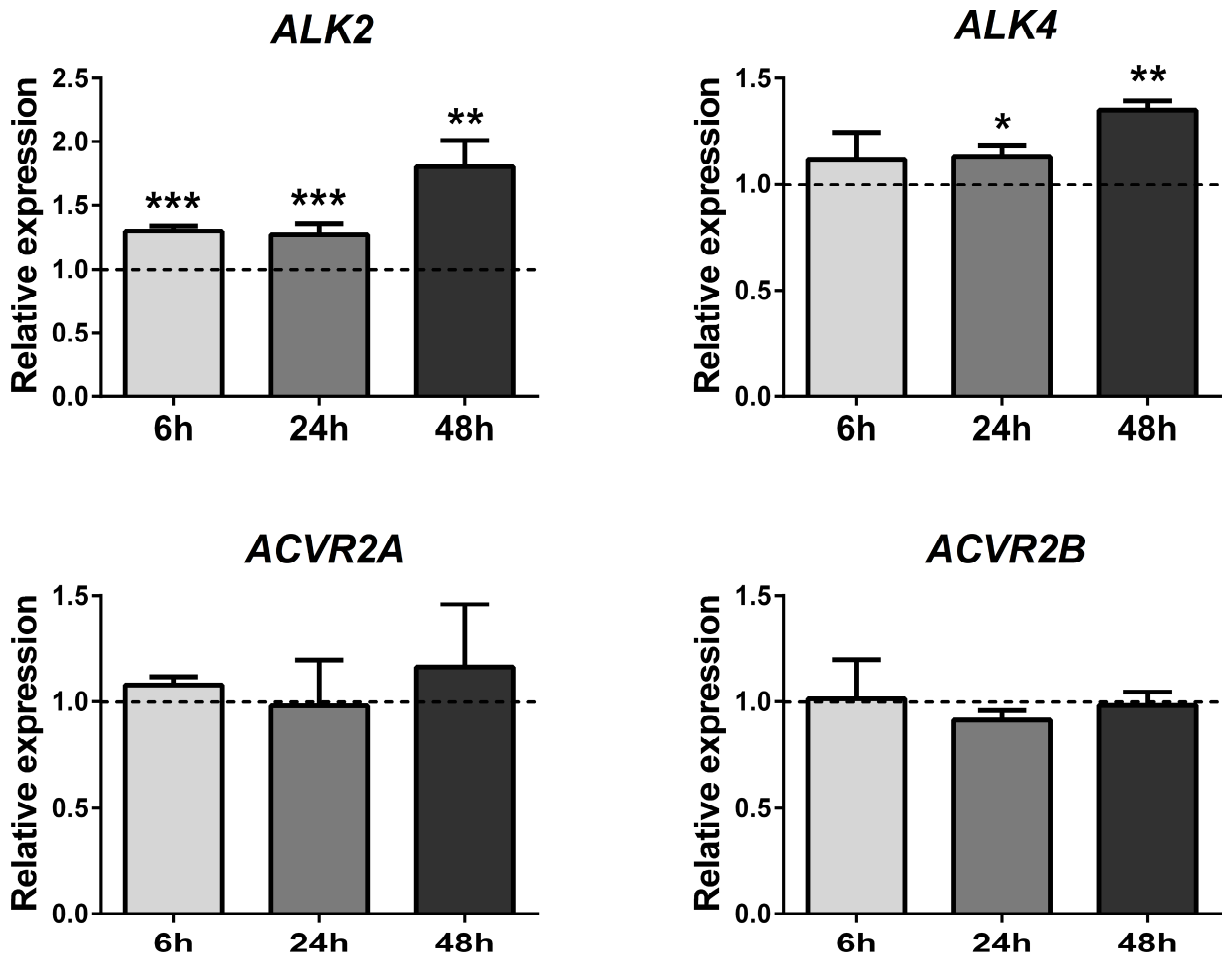
NALM-6 and 697 cells were stimulated or not with ActivinA (50 ng/mL) 24 hours before transplantation. Animals received i.v. 10^6 NALM-6 cells or 0.5×10^6 cells in 0.1 mL sterile PBS. Mice were sacrificed at two different timepoints (day +7 and +14 after 697 cell injection and day +11 and +14 after NALM-6 injection), and bone marrow, peripheral blood, liver, spleen, meninges and brain were analyzed for the presence of leukemic cells by flow cytometry. Engraftment was detected using anti-human CD19 APC-conjugated mAb (BD Pharmingen, CA, USA) and anti-human CD10 PE-Cy™7-conjugated mAb (BD Pharmingen) in the case of NALM-6 cells and anti-human CD45 PerCP-conjugated mAb (BD Pharmingen). PE-conjugated monoclonal antibody reacting with murine CD45 (eBioscience) was used to exclude murine hematopoietic cells. To ensure the specificity of the staining, Fc Receptor Binding Inhibitor Polyclonal Antibody (eBioscience) was used. Cells were analyzed with a FACS Canto II cytometer (BD Biosciences) and data analyzed by FlowJo software.

Supplementary results



Supplementary Figure 1. ALK2 was variably expressed by BCP-ALL cell lines and primary BM samples.

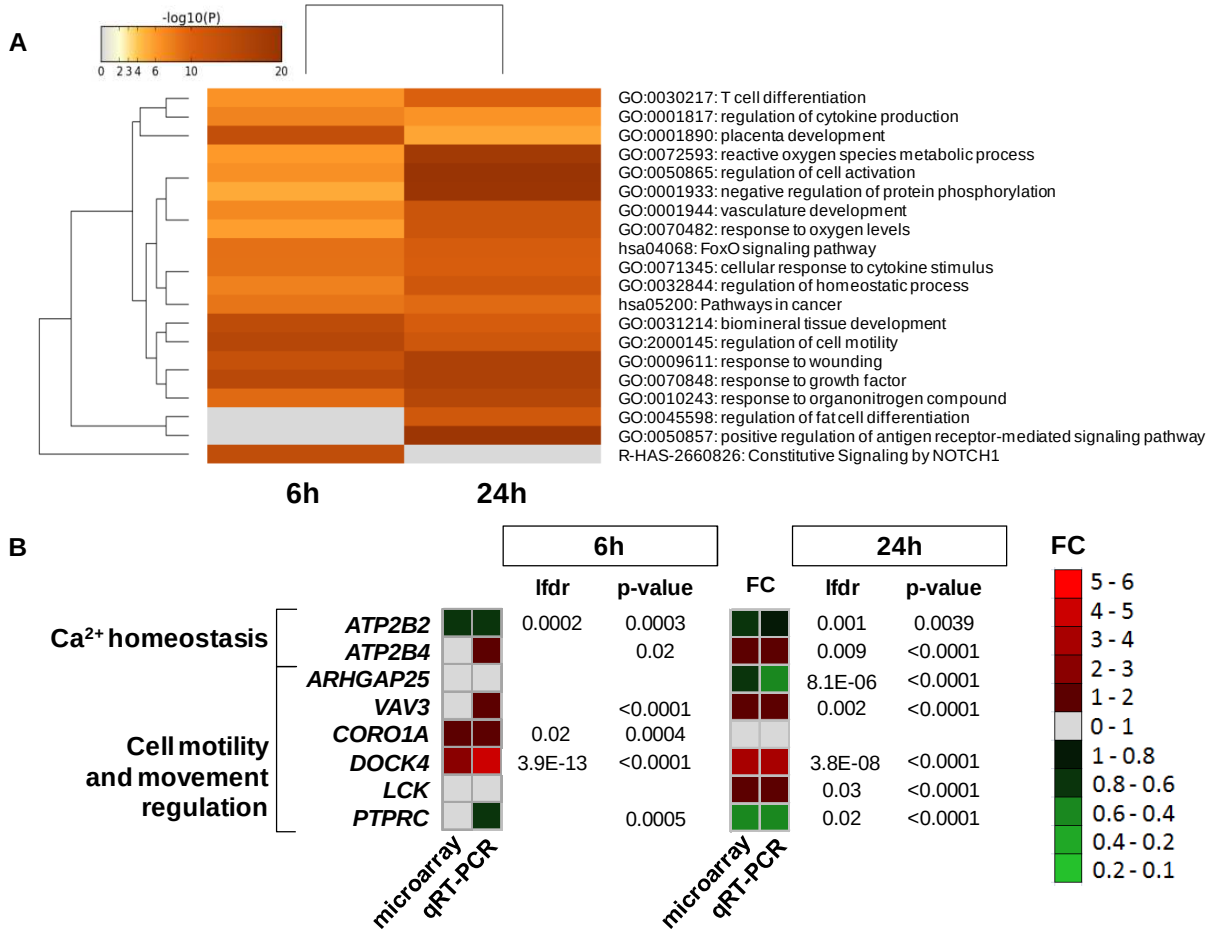
Western Blot analyses of ALK2 in five different BCP-ALL cell lines **(A)** and in primary blasts from twelve BCP-ALL patients **(B)** demonstrate a variable expression of the expected 57 KDa protein isoform. Additional bands with higher molecular weight, possibly derived from the glycosylation of the protein as already described in mouse cells[4], were further visualized. β-actin levels were used as a loading control.



Supplementary Figure 2. Modulation of ActivinA receptor expression by ActivinA.

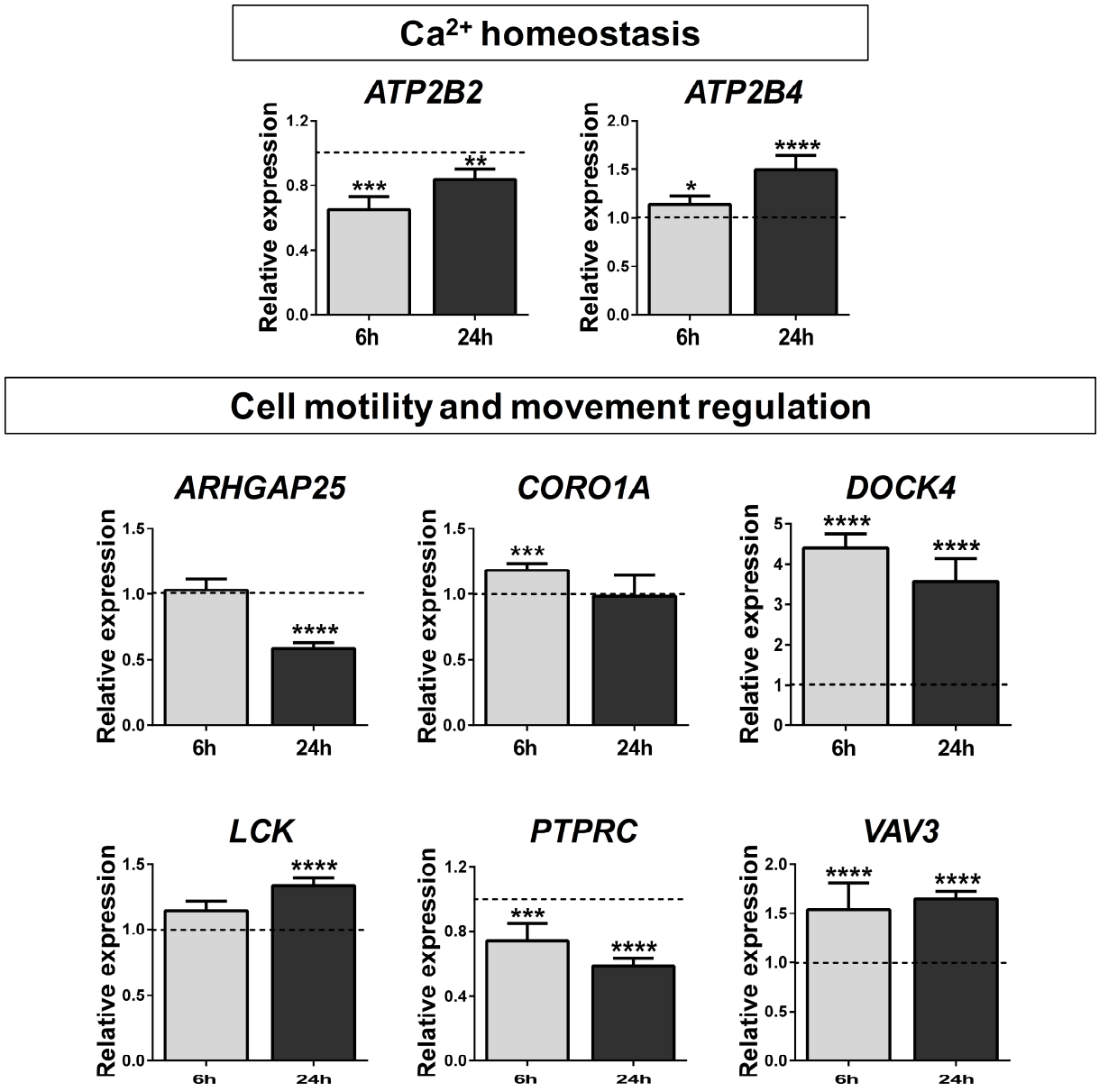
Expression of *ALK2*, *ALK4*, *ACVR2A* and *ACVR2B* was assessed in 697 cells treated or not with ActivinA (50 ng/mL) for 6 hours, 24 hours and 48 hours. The data are presented as the mean fold change ± SEM of mRNA levels normalized to *GAPDH* mRNA (endogenous control). The graphs represent the results of three independent experiments.

**P*<0.05, ***P*<0.01, ****P*<0.001: Mann-Whitney test.



Supplementary Figure 3. GEP analysis showed that ActivinA positively modulates cell motility in leukemic cells.

(A) Gene Ontology (GO) enrichment analysis of cell biological processes, based on differentially expressed genes in the 697 cell line treated or not with ActivinA (50 ng/mL) for 6 hours and 24 hours (n=4 independent experiments). The hierarchical clustering is based on the 20 most significant GO and KEGG terms resulting from Metascape analysis. In the heatmap colored cells were filled according their p-value. Grey cells indicate the lack of enrichment for that term in the corresponding gene list. **(B)** Selected genes significantly modulated in ActivinA treated cells compared to the untreated control were validated by qRT-PCR. Expression levels in qRT-PCR were normalized to GAPDH mRNA levels. Colored squares in the level plot correspond to FC between treatead *vs* untreated cells derived from microarray and qRT-PCR. Lfdr (Shrinkage *t* test) for microarray data and p-value (Mann-Whitney test) for qRT-PCR were respectively shown. FC= Fold Change; lfdr= local false discovery rate.

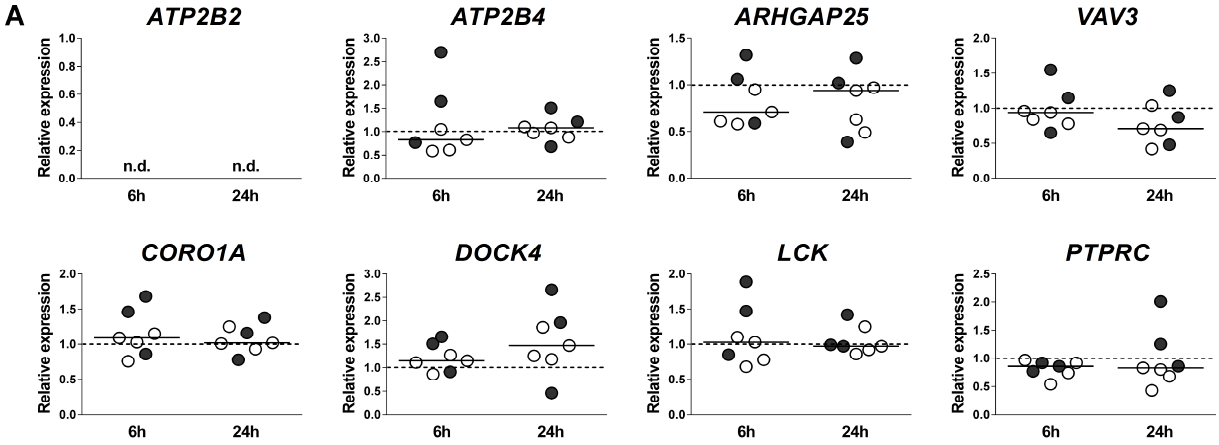


Supplementary Figure 4. Validation of gene expression analysis data.

Expression of *ATP2B2, ATP2B4, ARHGAP25, CORO1A, DOCK4, LCK, PTPRC* and *VAV3* was assessed in 697 cells treated or not with ActivinA (50 ng/mL) for 6 hours or 24 hours. The mean (± SEM, n=3 independent experiments) mRNA fold change calculated for each target gene in the ActivinA-stimulated condition over the unstimulated (dotted line), after normalization to *GAPDH* mRNA (endogenous control), is represented in the graph.

P*<0.05, ***P*<0.01, ****P*<0.001, **<0.0001: Mann-Whitney test.

A



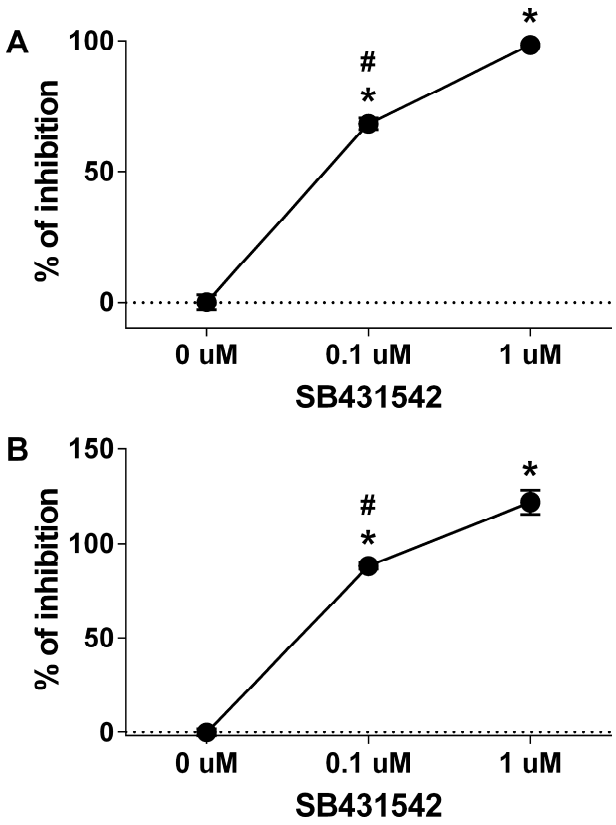
B

		negative*				t(1;19)		
		UPN6	UPN13	UPN14	UPN15	UPN2	UPN3	UPN12
ATP2B4	6 hrs	0,83	0,59	1,05	0,61	***2,70***	***1,65***	0,77
	24 hrs	1,10	0,88	0,97	1,08	***1,21***	***1,51***	0,68
ARHGAP25	6 hrs	***0,71***	***0,58***	0,95	***0,61***	1,32	1,06	***0,59***
	24 hrs	***0,49***	0,94	***0,63***	0,97	1,02	1,29	***0,39***
VAV3	6 hrs	0,94	0,78	0,96	0,84	***1,55***	***1,15***	0,65
	24 hrs	0,42	1,04	0,71	0,69	0,87	1,25	0,48
CORO1A	6 hrs	0,76	***1,15***	1,03	1,09	***1,68***	***1,46***	0,86
	24 hrs	***1,25***	1,02	0,93	1,01	0,78	***1,38***	***1,16***
DOCK4	6 hrs	***1,15***	0,86	1,11	***1,27***	***1,66***	***1,51***	0,91
	24 hrs	1,25	***1,86***	***1,47***	1,18	***2,66***	***1,96***	0,46
LCK	6 hrs	0,68	0,78	1,03	1,10	***1,89***	***1,47***	0,85
	24 hrs	1,25	0,92	0,97	0,86	0,97	***1,42***	0,99
PTPRC	6 hrs	0,97	***0,54***	0,92	***0,74***	0,92	***0,86***	***0,77***
	24 hrs	***0,43***	***0,80***	***0,68***	***0,83***	2,01	1,25	***0,86***

*t(4:11), t(9;22) p190, t(12;21), t(1;19)

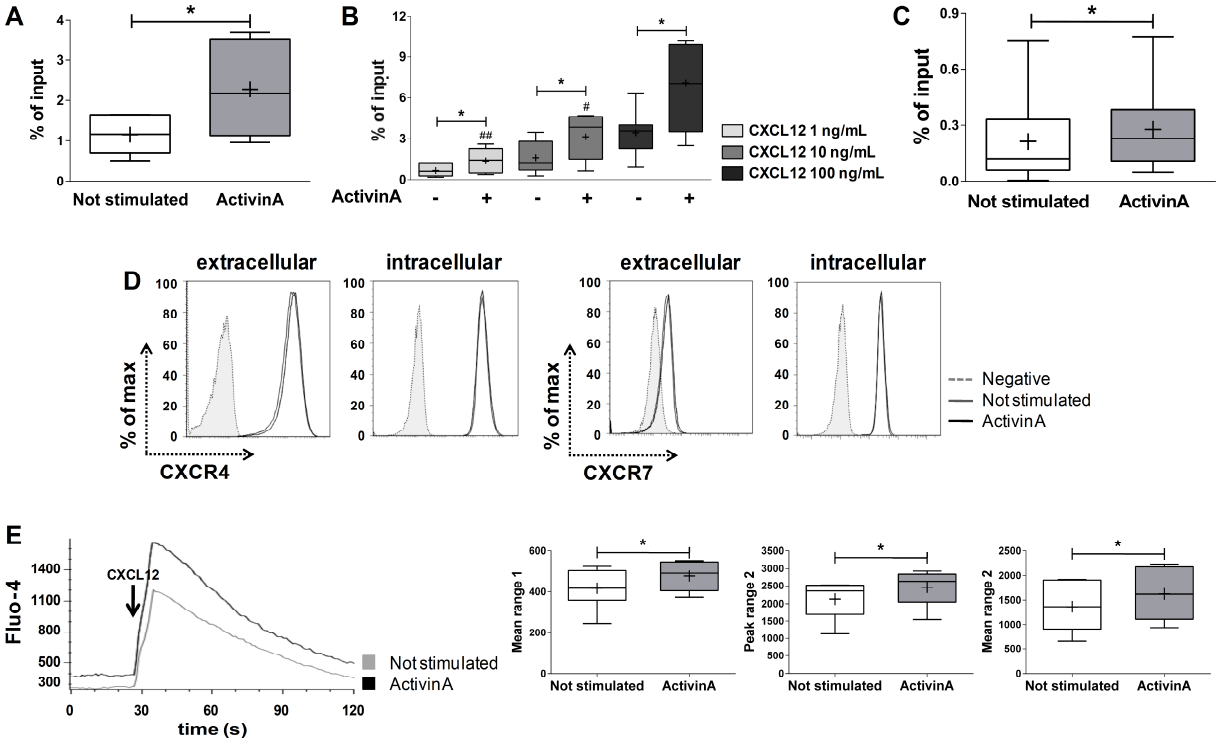
Supplementary Figure 5. Validation of gene expression analysis data on primary BCP-ALL blasts.

(A) Expression of *ATP2B2*, *ATP2B4*, *ARHGAP25*, *CORO1A*, *DOCK4*, *LCK*, *PTPRC* and *VAV3* was assessed in seven primary BCP-ALL patients treated or not with ActivinA (100 ng/mL) for 6 or 24 hours. The mean mRNA fold change calculated for each target gene in the ActivinA-stimulated condition over the unstimulated (dotted line), after normalization to *GAPDH* mRNA (endogenous control), is represented in the graph. Each dot represents a single patient: white circles indicate patients negative for t(4:11), t(9;22) p190, t(12;21), t(1;19), while grey circles indicate t(1;19) positive patients. Solid lines represent the median values. **(B)** Raw data about mean fold changes are reported in the table. Statistically significant changes (P<0.05, Mann-Whitney test) in ActivinA stimulated patients over the unstimulated condition are identified by "italics".



Supplementary Figure 6. SB431542 specifically inhibits ActivinA stimulatory effect on 697 and NALM-6 cell migration.

Chemotaxis assays were performed using 697 **(A)** and NALM-6 cells **(B),** pretreated for 1 hour with SB431542 or vehicle (DMSO), before the stimulation with or without ActivinA for 24 hours (50 ng/mL). Cells were allowed to migrate toward CXCL12-containing medium (100 ng/mL) for 4 hours (5 μM pores Transwell). The percentage of migrated cells was determined as described in Supplementary Methods. The average percentage of inhibition ± SEM is represented (one out of three representative experiments).

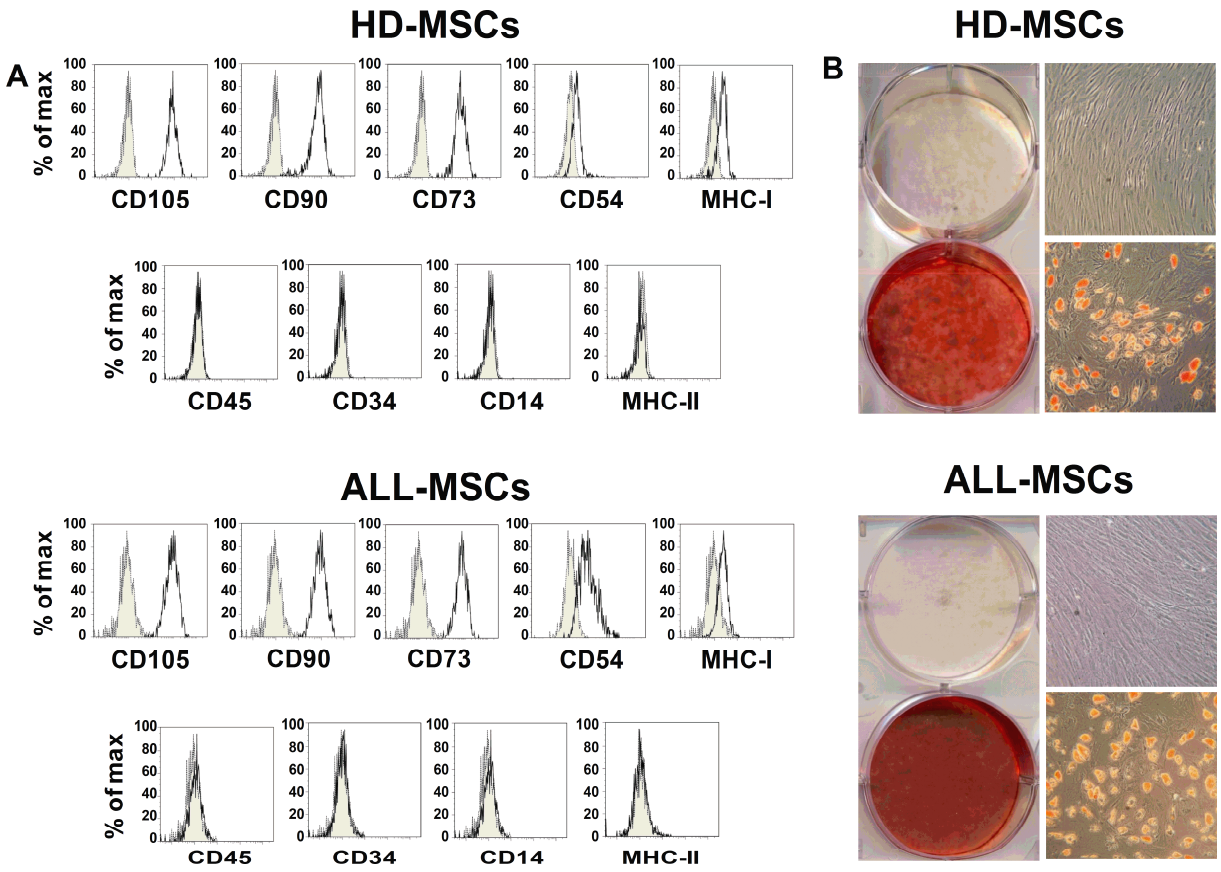


Supplementary Figure 7. ActivinA increased CXCL12-driven chemotaxis, invasion and calcium content in NALM-6 cells.

(A and B) Chemotaxis assay was performed using NALM-6 cells pretreated or not with ActivinA for 24 hours (50 ng/mL) and allowed to migrate toward CXCL12-containing medium for 4 hours. Each box plot shows the median and the mean (+) of the percentage of migrated cells and extends from the lowest to the highest value. The graphs represent the results of six independent experiments. **(C)** Invasion assay was set up using NALM-6 cells either stimulated or not with ActivinA for 24 hours (50 ng/mL) and allowed to migrate through Matrigel-coated (1 mg/mL) Transwell inserts for 24 hours in presence of CXCL12 (100 ng/mL). Each box plot shows the median and the mean (+) of the percentage of invaded cells and extends from the lowest to the highest value. The graphs represent the results of six independent experiments. **(D)** The extracellular and intracellular CXCR4 and CXCR7 expression in stimulated (black line) or unstimulated cells (grey line) with ActivinA for 24 hours was evaluated by flow cytometry. Data from one out of three independent experiments are shown. **(E)** NALM-6 cells were cultured for 24 hours with or without ActivinA (50 ng/mL). Cells were incubated with Fluo-4 NW dye and cytosolic free Ca^{2+} changes were measured by flow cytometry. The left panel shows a representative calcium flux profile of NALM-6 cells. The black line represents ActivinA-treated cells, while the grey line corresponds to untreated cells. The arrow indicates CXCL12 addition. In the right panels, each box plot shows the median and the mean (+) and extends from the lowest to the highest value. The three plots represent the MFI before (Mean Range 1) and after (Mean Range

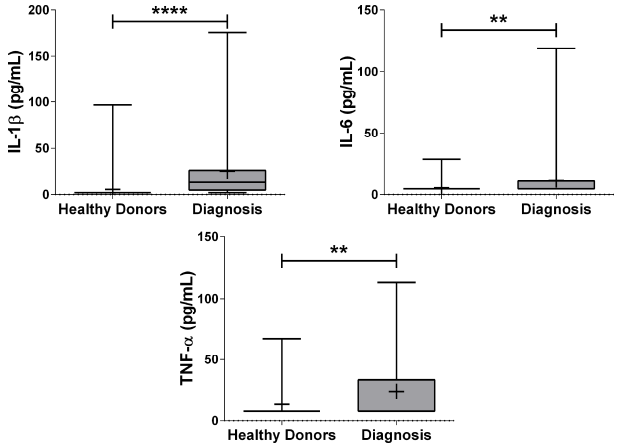
2) the addition of CXCL12 and the maximum peak reached upon CXCL12 addition (Peak Range 2). The graphs represent the results of six independent experiments.

*P<0.05: Wilcoxon matched-pairs signed rank test (Panels A, B, C and E); #P<0.05, ##P<0.01: comparison with 100 ng/mL CXCL12-induced migration, Mann-Whitney test (Panel B).



Supplementary Figure 8. ALL-MSCs were similar to HD-MSCs in terms of phenotype and differentiation capacity.

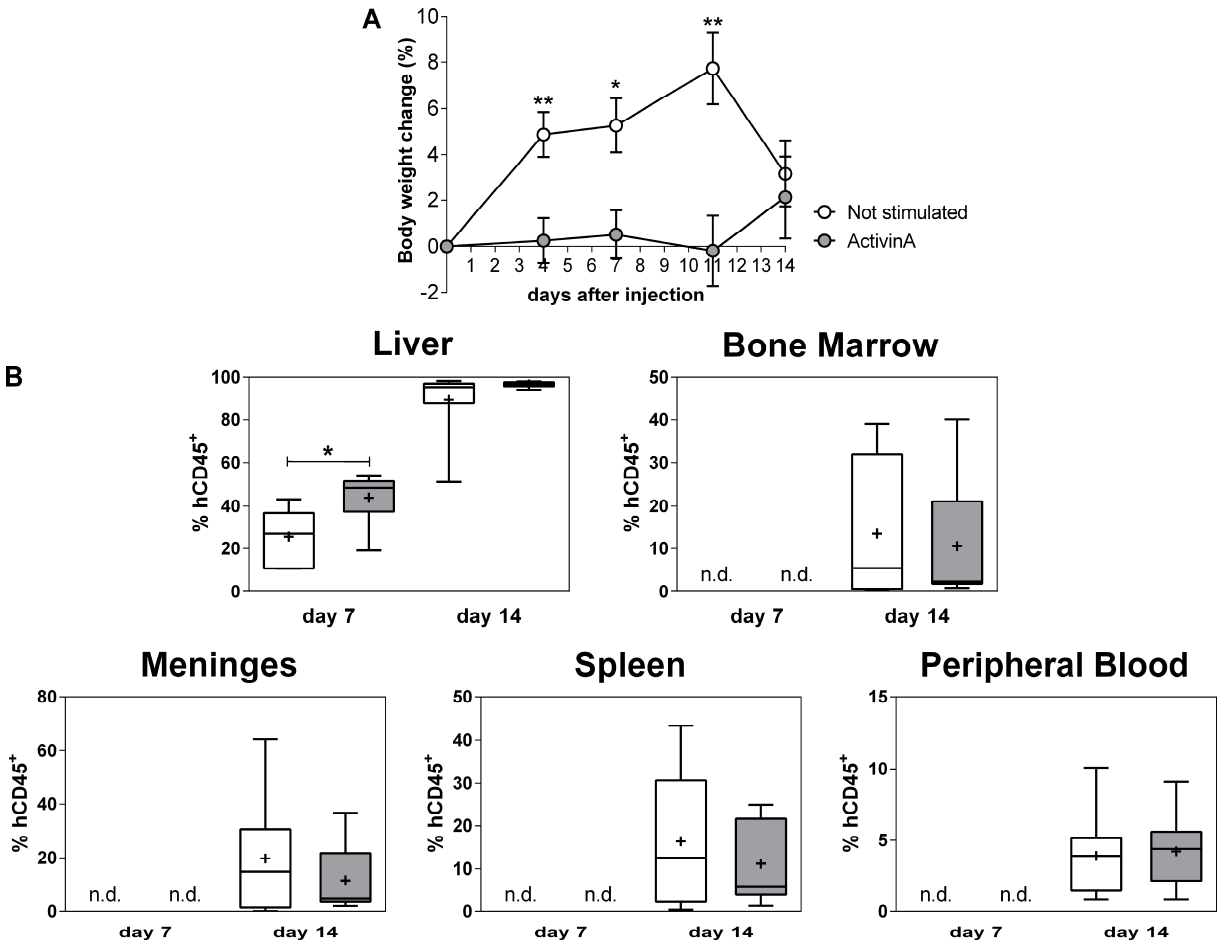
(A) Immunophenotype of both HD-MSCs and ALL-MSCs was analyzed by flow cytometry. Mesenchymal stromal cells were positive for typical mesenchymal markers including CD105, CD90, CD73, CD54 and MHC-I, while lacked the expression of the markers CD14, CD34, CD45 and MHC-II. **(B)** HD-MSCs and ALL-MSCs were induced to differentiate toward osteogenic (left Panel) and adipogenic lineage (right Panel), as shown by Alizarin Red staining of calcium deposits and Oil Red O lipophilic dye, respectively.



Supplementary Figure 9. Pro-inflammatory cytokine levels were higher in the leukemic BM microenvironment compared to HDs.

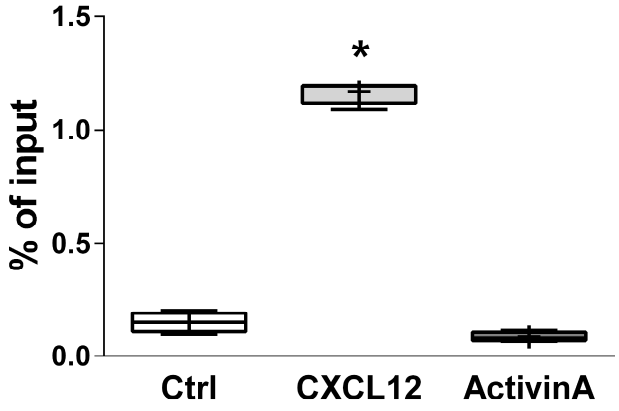
IL-1β, IL-6 and TNF-α BM plasma levels were assessed by ELISA in HDs and BCP-ALL patients at the onset of the disease. Each box plot shows the median and the mean (+) and extends from the lowest to the highest value.

*P<0.05, ** P<0.01, ****P<0.0001: Mann-Whitney test.



Supplementary Figure 10. ActivinA enhanced the engraftment of 697 cells to the liver of NSG mice

(A) NSG mice were i.v. injected with 0.5×10^6 697 cells, previously stimulated or not for 24 hours with ActivinA (50 ng/mL). Body weight change was periodically monitored over 2 weeks after transplantation. Mean ± SEM of body weight change (%) from three independent experiments (9 mice/group) is shown. **P*<0.05, ***P*<0.01: Mann-Whitney test. **(B)** Mice were sacrificed on days 7 and 14 after transplantation and the percentage of infiltrating $hCD45^+$ leukemic cells was determined by flow cytometry in the liver, bone marrow, meninges, spleen, peripheral blood. Each box plot shows the median and the mean (+) and extends from the lowest to the highest value. **P*<0.05: Mann-Whitney test.



Supplementary Figure 11. ActivinA did not induce 697 cellular chemotaxis.

697 cells were allowed to migrate for 4 hours toward empty medium (Ctrl) or toward CXCL12 (100 ng/mL, positive control) or ActivinA (50 ng/mL) added to the lower chamber. The graph represents one experiment in triplicate. Each box plot shows the median and the mean (+) and extends from the lowest to the highest value.

*$P<0.05$: Mann-Whitney test *versus* control.

Supplementary references

1. Irizarry RA, Hobbs B, Collin F, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics (Oxford, England). 2003;4(2):249-264.
2. Opgen-Rhein R, Strimmer K. Accurate Ranking of Differentially Expressed Genes by a Distribution-Free Shrinkage Approach. Statistical applications in genetics and molecular biology. 2007;6(1):Article9.
3. Tripathi S, Pohl MO, Zhou Y, et al. Meta- and Orthogonal Integration of Influenza "OMICs" Data Defines a Role for UBR4 in Virus Budding. Cell host & microbe. 2015;18(6):723-735.
4. Rosendahl A, Pardali E, Speletas M, Ten Dijke P, Heldin CH, Sideras P. Activation of bone morphogenetic protein/Smad signaling in bronchial epithelial cells during airway inflammation. American journal of respiratory cell and molecular biology. 2002;27(2):160-169.