Physical contact with endothelial cells through β_1- and β_2- integrins rescues chronic lymphocytic leukemia cells from spontaneous and drug-induced apoptosis and induces a peculiar gene expression profile in leukemic cells

Rossana Maffei,[1] Stefania Fiorcari,[1] Jenny Bulgarelli,[1] Silvia Martinelli,[1] Ilaria Castelli,[1] Silvia Deaglio,[2] Giulia Debbia,[1] Marcella Fontana,[1] Valeria Coluccio,[1] Goretta Bonacorsi,[1] Patrizia Zucchini,[1] Franco Narni,[1] Giuseppe Torelli,[1] Mario Luppi,[1] and Roberto Marasca[1]

[1]Hematology Unit, Department of Oncology, Hematology and Respiratory Diseases, University of Modena and Reggio Emilia, Modena; and [2]Department of Genetics, Biology and Biochemistry, University of Turin and Human Genetics Foundation, Turin, Italy

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Online Supplementary Appendix

Cell culture conditions

Human umbilical vein endothelial cells (HUVEC, Cascade Biologics, Invitrogen, Portland, OR, USA) pooled from multiple isolates were cultured in M200 PRF medium supplemented with a Low serum Growth Supplement Kit (Cascade Biologics) obtaining a final concentration of 2% fetal bovine serum, 1 μg/mL hydrocortisone, 10 ng/mL human epidermal growth factor, 3 ng/mL basic fibroblast growth factor and 10 μg/mL heparin. When HUVEC cultures reached 80% confluence, cells were detached with trypsin/EDTA solution and seeded at a concentration of 1.25×10^4 viable cells/mL in tissue culture flasks coated with Attachment Factor (Cascade Biologics) containing gelatin at 0.1%. HUVEC were used at passage 2-4 in all experiments.

Peripheral blood mononuclear cells, taken at the time of diagnosis, were isolated by density gradient centrifugation (Ficoll, Pharmacia LKB Biotechnology, Piscataway, NY, USA) and cryo - preserved in RPMI-1640 medium, 50% fetal bovine serum, and 10% dimethylsulfoxide and stored in liquid nitrogen until use. To enrich for chronic lymphocytic leukemia (CLL) cells, the peripheral blood mononuclear cells were incubated with CD19Microbeads (Miltenyi Biotech, Auburn, CA, USA), obtaining a purity >99% as assessed by flow cytometry using phycoerythrin-conjugated CD19 (Miltenyi Biotech). To evaluate the effect of positive cell selection, we compared survival rates of CLL cells either isolated by positive selection or enriched by depletion of non-B cells (B-cell isolation kit – BCLL -Miltenyi Biotech), and observed no differences.

Purified $CD19^+$ CLL cells were suspended at a final concentration of 1×10^6/mL in AIM-V medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum and then plated in 24-well plates alone (CLL only) or on HUVEC layers (CLL HC). At the indicated time points, CLL cells were collected by removal of the supernatant leaving the HUVEC intact and then assayed for cell viability and gene expression profiling. About 97-98% of all CLL cells were recovered by pipetting the well, whereas 2-3% of cells remained tightly adherent to HUVEC or underneath the monolayer, as evaluated by flow cytometric counting of lymphocytes.

Conditioned media were collected by centrifugation at 1600 rpm for 10 min and stored at -20°C before being assayed or used to stimulate CLL cells cultured alone (dilution 1:2 with fresh medium). In parallel experiments CLL cells were physically separated from HUVEC using Transwell Inserts (BD Biosciences, Milan, Italy) with a pore size of 0.4 μm.

In some experiments, leukemic cells were cultured in medium with addition of 10 μM fludarabine (2-fluoroadenine 9-B-D-arabinofuranoside, Sigma, Milan, Italy). For inhibition studies, cells were incubated for 1 h with the following blocking antibodies at 10 μg/mL: anti-CD11a (clone 25.3; Immunotech, Marseille, France), anti-CD18 (clone L130; BD Biosciences), anti-CD49d (clone HP2/1; Immunotech) and anti-CD29 (clone LIa1/2; Immunotech) for CLL and anti-CD106 (clone 51-10C9; BD Biosciences) and anti-CD54 (clone BBIG-I1; R&D Systems, Minneapolis, MN, USA) for HUVEC before co-culture. An isotypic antibody (IgG, clone 11711, R&D Systems) was added as an irrelevant control.

Flow cytometry and confocal microscopy

Flow cytometric data were obtained using a FACSCalibur cytometer (Becton Dickinson) and then analyzed by CellQuest Software (Becton Dickinson). Data are presented as the mean ± standard error of mean (SEM).

For confocal microscopy studies, CLL HC layers were fixed with paraformaldehyde (4% in phosphate-buffered saline), permeabilized using saponin (0.1% in phosphate-buffered saline) and saturated using non-immune goat serum.

Slides were analyzed using a Leica TCSSP5 confocal microscope at 63x magnification. Images were acquired using LAS AF (Leica, Milan, Italy) and processed using Photoshop (San Jose, CA, USA) software.

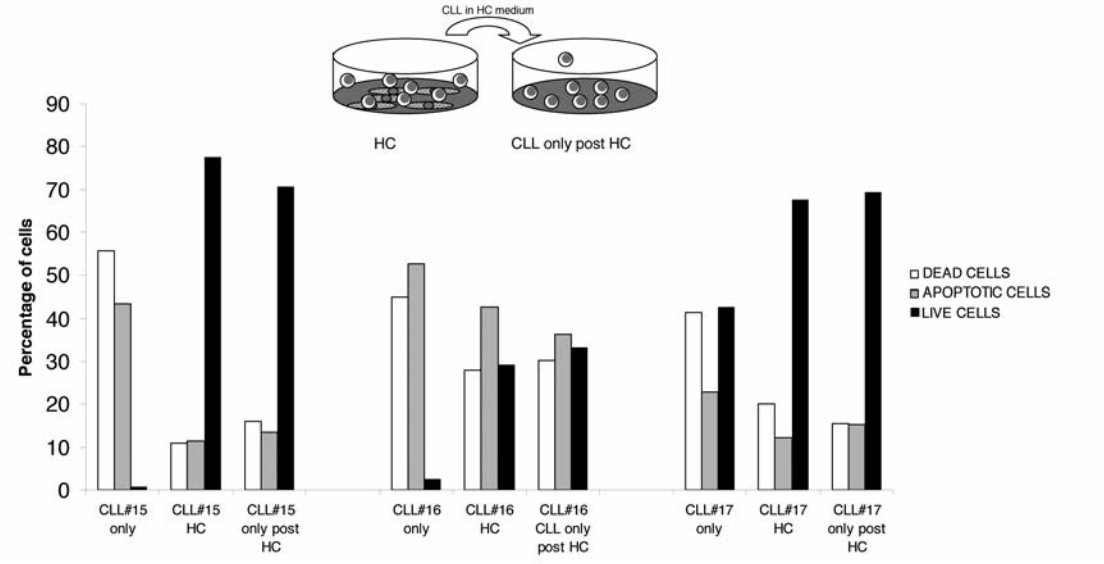
Genome-wide expression profiling

For genome-wide expression profiling (GEP) analysis, all samples collected from co-culture conditions were investigated by flow cytometry using phyoerythrin-conjugated CD19 (Miltenyi Biotech) to exclude HUVEC contamination. High quality RNA were amplified and labeled with cyanine-3 (Cy3) using the Low Input Quick Amp Labeling kit (Agilent Technologies, Palo Alto, CA, USA). Agilent RNA One-Color Spike-In was added to each

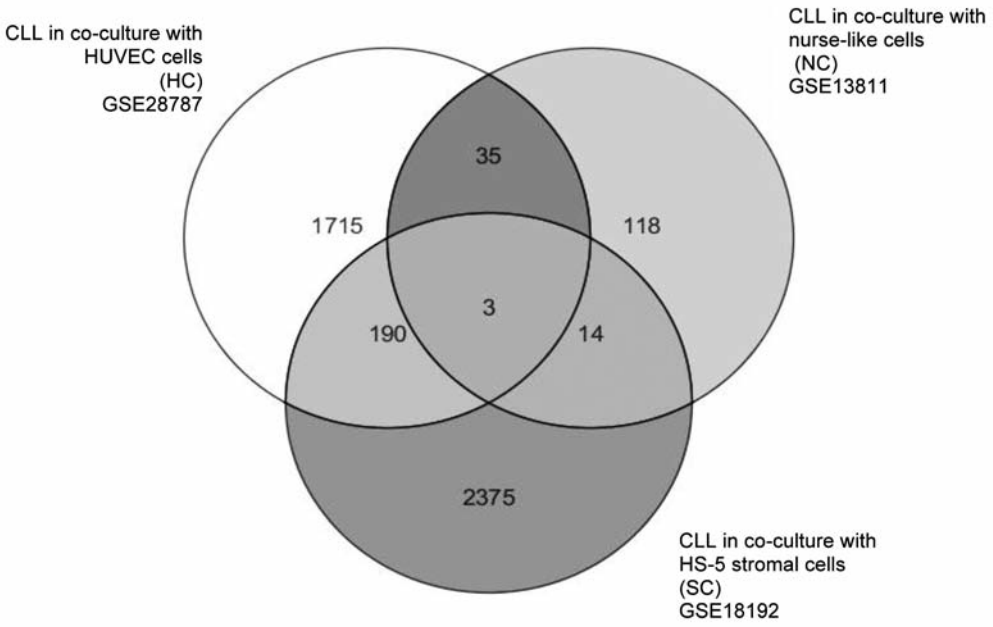
sample to provide positive controls for monitoring the microarray workflow from sample amplification and labeling to microarray processing. All cRNA products were purified using RNeasy columns (QIAGEN). Samples had to contain at least 6 pmol of cyanine dye/μg of cRNA to be considered suitable for subsequent hybridization. Cy3-labeled cRNA (1.65 μg) were fragmented to an average size of 50-100 nt by incubation at 60°C for 30 min using an *in situ* Hybridization kit-plus (Agilent). Samples were hybridized for 17 h at 65°C on 4×44K Whole Human Genome Microarray (Agilent) and then scanned using a laser scanner (Agilent Technologies). Fluorescence data were analyzed with Feature Extraction Software v.10.5 (Agilent Technologies) and QC Chart tool v.1.3. Agglomerative two-dimensional clustering analysis and supervised analyses based on t-testing were performed using Gene Spring GX (Agilent) software. Genes were defined as differentially expressed between groups at a significance level of $P<0.05$ and with a fold change cut off ± 2 in all the pair wise comparisons. Gene Ontology Tool (*http://www.geneontology.org/*) and PANTHER Classification System (Protein ANalysis THrough Evolutionary Relationships, *http://www.pantherdb.org/*) were used to unravel biological function and pathways represented in the gene lists.

Immunohistochemistry

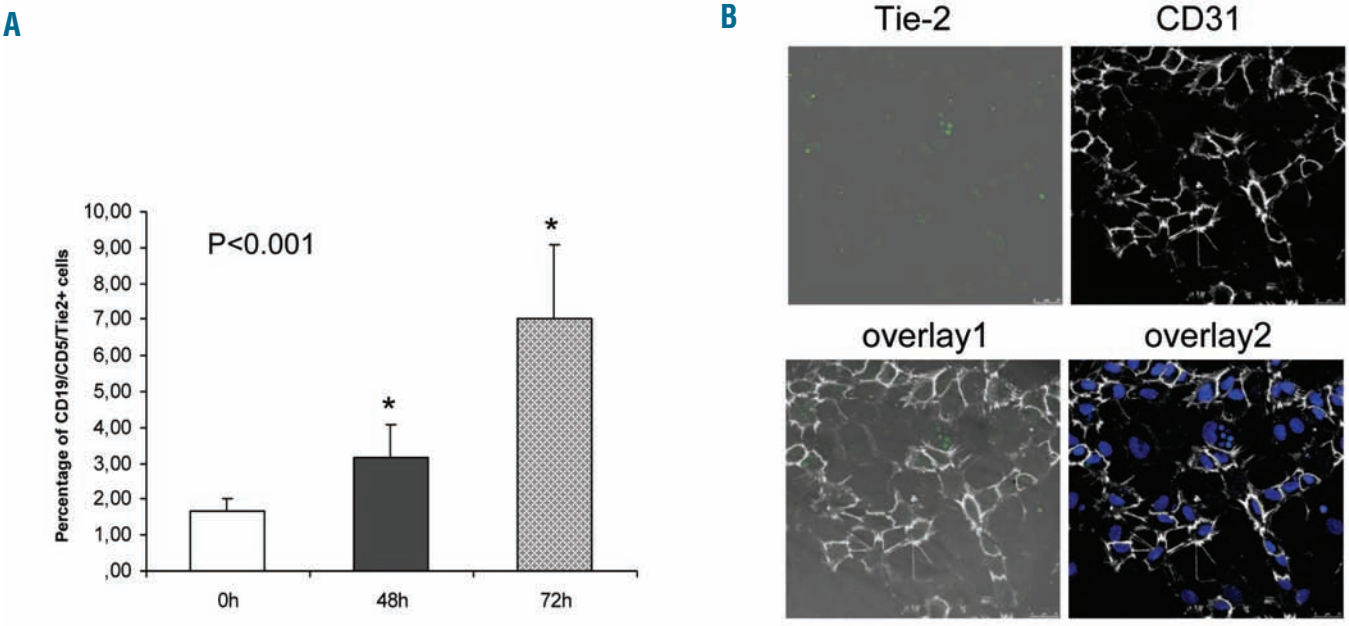
Lymph node biopsies were incubated for 30 min with the primary monoclonal mouse antibody anti-endothelin1 (Calbiochem, Merck, Darmstadt, Germany, dilution 1:250) and with the primary monoclonal antibody mouse anti–human angiopoietin-2 (MAB0983, R&D Systems, dilution 1:50). Bound antibody was detected with the alkaline phosphatase method using a streptavidin-biotin alkaline phosphatase complex kit (Dako REAL™ Detection System, Alkaline Phosphatase/RED, Rabbit/Mouse, DAKO Dako, Glostrup, Denmark). The alkaline phosphatase reaction was then developed with Permanent Red (DAKO) as the chromogen and counterstained with hematoxylin.



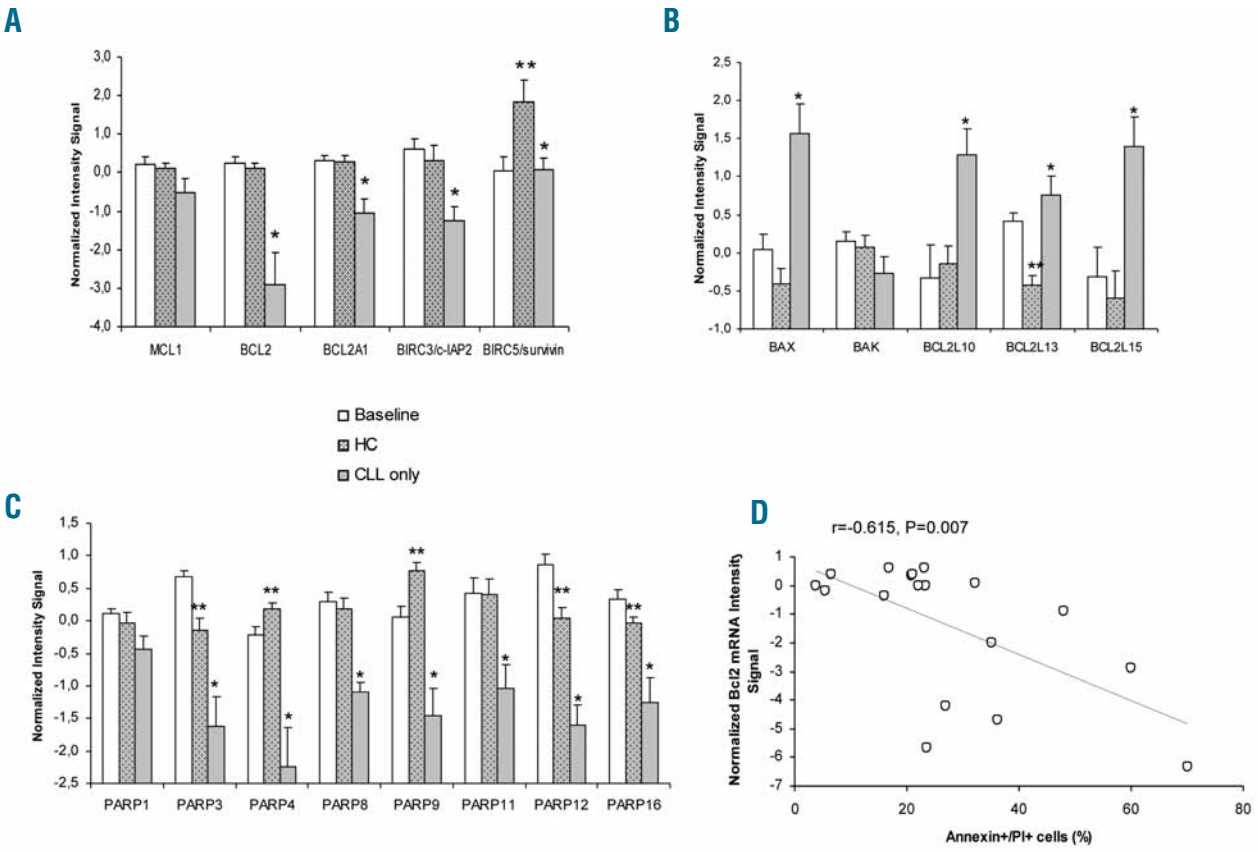
Online Supplementary Figure S1. **CLL survival advantage in an EC co-culture is partially mediated by soluble factors. CLL cells were cultured in medium alone (CLL only) or on a HUVEC layer (HC) for 96 h. In addition, as depicted in the above schematic cartoon, CLL were cultured on a HUVEC layer for 48 h and then CLL cells were separated from the EC and cultured for a further 2 days alone in the same HC medium (CLL only post HC). Data are presented as percentages of dead, apoptotic and live cells for three patients at 96 h. Note that HC-stimulated CLL maintained increased viability even if separated from the EC layer.**



Online Supplementary Figure S2. Venn diagram comparing three sets of differentially expressed genes (all FC=2, *P*<0.05) in CLL co-cultured on HUVEC (HC) , on nurse-like cells (NC) and on HS-5 stromal cells (SC). Few genes were shared by HC, NC and SC culture conditions.



Online Supplementary Figure S3. (A) Flow cytometric evaluation of Tie2 receptor on CLL cells (n=8) co-cultured for 3 days on an EC layer. Percentages of $CD19^+/CD5^+/Tie2^+$ cells are significantly increased by EC contact. **P*<0.05 compared to baseline (Wilcoxon's test). (B) CLL cells were co-cultured for 48 h on HUVEC, and non-adherent cells were removed before confocal immunofluorescence analysis for Tie2 (green), CD31 (white) and DAPI (blue). Confocal planes obtained at 63x magnification are shown individually and after merging. The expression of Tie2 was detected on a fraction of adherent CLL cells.



Online Supplementary Figure S4. CLL cells maintain or increase expression of anti-apoptotic factors in co-culture conditions. mRNA levels in CLL cells at baseline, after 2 days of co-culture with HUVEC and after 2 days of culture alone of some anti-apoptotic (A), pro-apoptotic factors (B) and PARP (C). Histograms represent microarray normalized intensity signals. *P<0.05 in CLL only compared to HC, **P<0.05 in HC compared to CLL at baseline. (D) A negative correlation is present between Bcl2 mRNA levels and the percentage of annexin$^+$/propidium iodide$^+$ cells for the same patient.

Online Supplementary Table S1. GO categories of genes in CLL cells modulated by co-culture with an endothelial cell layer.

GO ACCESSION	GO Term	p-value
GO:0001568	blood vessel development	1.0E-06
GO:0048514	blood vessel morphogenesis	1.0E-06
GO:0001944	vasculature development	1.0E-06
GO:0001525	angiogenesis	9.3E-06
GO:0048646	anatomical structure formation	1.5E-05
GO:0051270	regulation of cell motion	4.3E-04
GO:0030334	regulation of cell migration	4.9E-04
GO:0009887	organ morphogenesis	4.9E-04
GO:0042060	wound healing	5.8E-04
GO:0001569	patterning of blood vessels	6.1E-03
GO:0050793	regulation of developmental process	6.1E-03
GO:0009968	negative regulation of signal transduction	1.2E-02
GO:0046872	metal ion binding	1.7E-02
GO:0005925	focal adhesion	1.7E-02
GO:0006937	regulation of muscle contraction	2.2E-02
GO:0008360	regulation of cell shape	2.2E-02
GO:0050878	regulation of body fluid levels	2.3E-02
GO:0005924	cell-substrate adherens junction	2.5E-02
GO:0043167	ion binding	2.5E-02
GO:0065007	biological regulation	2.5E-02
GO:0007596	blood coagulation	2.8E-02
GO:0050817	coagulation	2.8E-02
GO:0043169	cation binding	2.8E-02
GO:0009966	regulation of signal transduction	4.6E-02
GO:0030055	cell-substrate junction	4.6E-02
GO:0006950	response to stress	5.0E-02
GO:0046914	transition metal ion binding	5.2E-02
GO:0007599	hemostasis	5.6E-02
GO:0022604	regulation of cell morphogenesis	6.2E-02
GO:0009605	response to external stimulus	7.4E-02
GO:0009653	anatomical structure morphogenesis	7.5E-02
GO:0001666	response to hypoxia	8.6E-02
GO:0048513	organ development	8.6E-02
GO:0050789	regulation of biological process	8.9E-02
GO:0030036	actin cytoskeleton organization and biogenesis	9.2E-02
GO:0032502	developmental process	9.8E-02
GO:0048661	positive regulation of smooth muscle cell proliferation	9.8E-02

Online Supplementary Table S2. Differentially expressed genes in CLL cells co-cultured on an endothelial layer.

Cellular Pathway	Up/ down	Gene symbol	Gene name	Fold change	p-value
TGFβ signaling	UP	TGFBR2	TGF-beta receptor type-2	2.3	0.00276
	UP	TGFBR1	TGF-beta receptor type-1	2.6	0.01437
	UP	ACVR1	Activin receptor type-1	2.8	0.01160
	UP	ACVRL1	Serine/threonine-protein kinase receptor R3	30.7	0.00580
	UP	ENG	Endoglin	4.0	0.02540
	UP	CD109	CD109 antigen	11.7	0.01829
	UP	BMP4	Bone morphogenetic protein 4	8.3	0.02216
	UP	GDF15	Growth/differentiation factor 15	18.6	0.00073
	UP	INHBA	Inhibin beta A chain	11.4	0.03279
	UP	ZFYVE16	Zinc finger FYVE domain-containing protein 16	2.4	0.00496
	UP	SMAD7	Mothers against decapentaplegic homolog 7	14.0	0.00140
	UP	SMAD1	Mothers against decapentaplegic homolog 1	6.6	0.02538
	UP	FOSL1	Fos-related antigen 1	2.5	0.04631
	UP	SKIL	Ski-like protein	4.0	0.00014
	UP	ID1	DNA-binding protein inhibitor ID-1	14.7	0.00098
	DOWN	CITED1	Cbp/p300-interacting transactivator 1	-2.6	0.00821
Wnt signaling	UP	FZD4	Frizzled-4	16.1	0.0026
	UP	FZD5	Frizzled-5	5.3	0.0044
	UP	FZD7	Frizzled-7	4.3	0.0060
Angiogenesis	UP	TIE2	Angiopoietin-1 receptor	10.7	0.0170
	UP	TIE1	Tyrosine-protein kinase receptor Tie-1	18.9	0.0088
	UP	VEGFC	Vascular endothelial growth factor C	9.4	0.0061
	UP	CCL2	C-C motif chemokine 2	22.6	0.0032

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	UP	AMOTL2	Angiomotin-like protein 2	4.3	0.0191
	UP	ANGPTL4	Angiopoietin-related protein 4	8.6	0.0154
	UP	EDN1	Big endothelin-1	9.2	0.0061
	UP	THBS-1	Thrombospondin-1	45.1	0.0004
	DOWN	FLT4	Vascular endothelial growth factor receptor 3	-2.1	0.0048
Response to hypoxia	UP	HIF1A	Hypoxia-inducible factor 1 alpha	3.9	0.0111
	UP	HIF2A/EPAS1	Endothelial PAS domain-containing protein 1	12.9	0.0067
	UP	HIF3A	Hypoxia-inducible factor 3 alpha	2.2	0.0330
Integrin signaling	UP	ITGA2	Integrin alpha-2	5.2	0.0219
	UP	ITGA6	Integrin alpha-6 light chain	6.7	0.0213
	UP	ACTN1	Alpha-actinin-1	11.9	0.0067
	UP	CAV1	Caveolin-1	18.9	0.0163
	UP	COL8A1	Collagen alpha-1(VIII) chain	15.8	0.0025
	UP	COL9A3	Collagen alpha-3(IX) chain	2.9	0.0413
	UP	COL5A1	Collagen alpha-1(V) chain	4.0	0.0173
	UP	COL12A1	Collagen alpha-1(XII) chain	5.4	0.0371
	DOWN	ITGB7	Integrin beta-7	-2.4	0.0047
Cell adhesion	UP	CLDN12	Claudin-12	4.1	0.0100
	UP	CLDN22	Claudin-22	4.2	0.0383
	UP	CLDN5	Claudin-5	14.5	0.0041
	DOWN	CLDN11	Claudin-11	-2.2	0.0177
	UP	CDH11	Cadherin-11	9.7	0.0293
	UP	CDH13	Cadherin-13	7.1	0.0297
	UP	CDH5	Cadherin-5	5.1	0.0304
	DOWN	CDH23	Cadherin-23	-2.1	0.0038
	UP	GJA1	Gap junction alpha-1 protein	18.4	0.0136

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	UP	PLXNB2	Plexin-B2	5.7	0.0311
	UP	PLXND1	Plexin-D1	6.9	0.0025
Others	UP	CCR6	C-C chemokine receptor type 6	7.3	0.0050
	UP	ABCB1	Multidrug resistance protein 1	6.3	0.0445
	UP	PDGFC	Platelet-derived growth factor C, receptor-binding form	6.5	0.0051

** Up- or down-regulated genes in CLL cells co-cultured for 48 h on an endothelial layer compared to CLL cells at baseline*