Mixed-species RNAseq analysis of human lymphoma cells adhering to mouse stromal cells identifies a core gene set that is also differentially expressed in the lymph node microenvironment of mantle cell lymphoma and chronic lymphocytic leukemia patients

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SUPPLEMENTARY FIGURES

Supplementary Figure 1

A

B
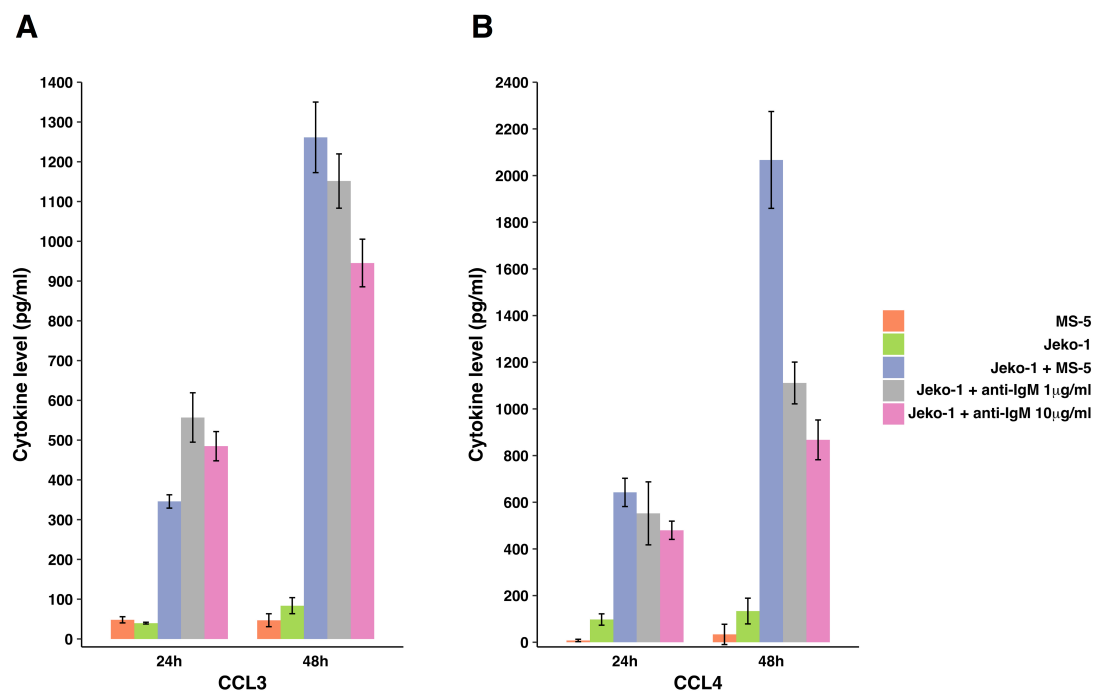
**Supplementary Figure 1. CCL3 and CCL4 cytokine levels increase in conditioned media following 24h and 48h co-culture of Jeko-1 cells with MS-5 stromal cells, induction level changes are of a similar magnitude to those following B cell receptor stimulation by anti-IgM for 24h and 48h.**

Cytokine levels for CCL3 and CCL4 in conditioned media following 24h and 48h co-culture of Jeko-1 cells with MS-5 stromal cells (blue bars) compared to cytokine levels following B cell receptor stimulation of Jeko-1 cells by anti-IgM for 24 and 48h (grey and pink bars) as measured by sandwich ELISA. Mono-cultured untreated MS-5 cells in red and mono-cultured untreated Jeko-1 cells in green.
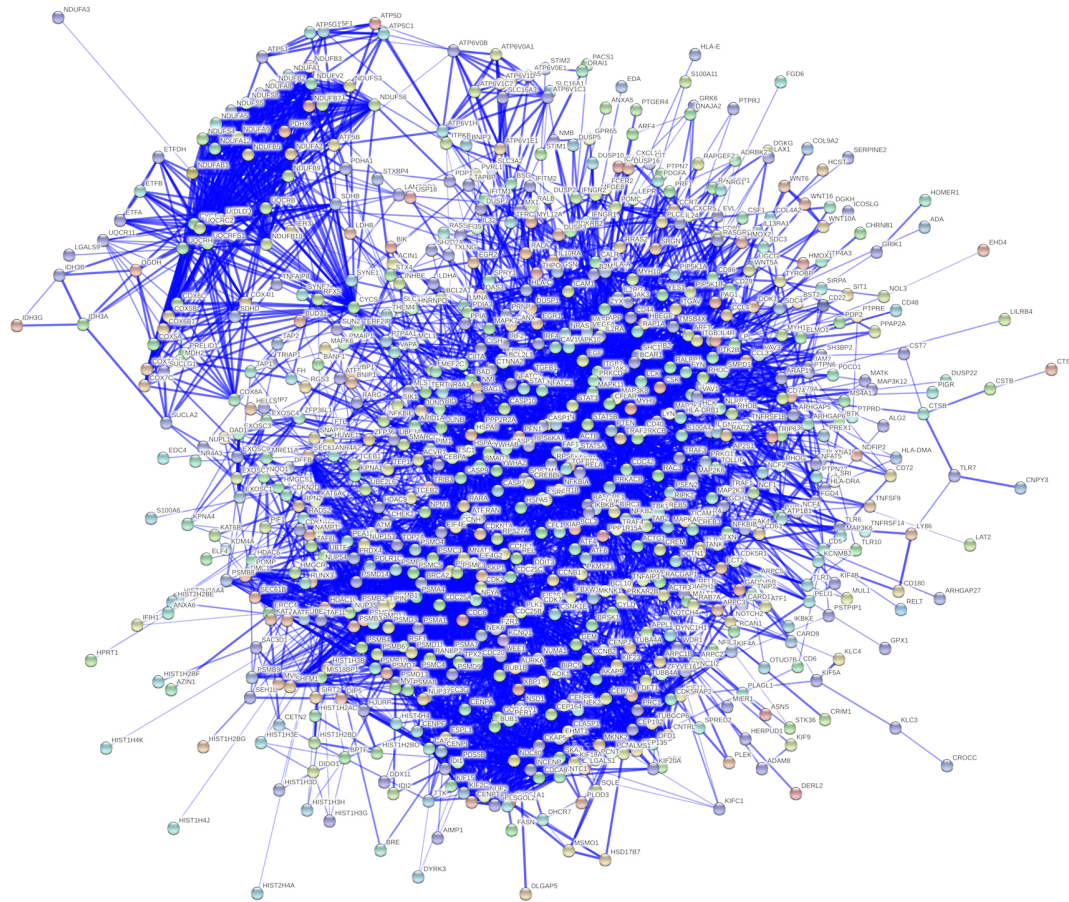
Supplementary Figure 2
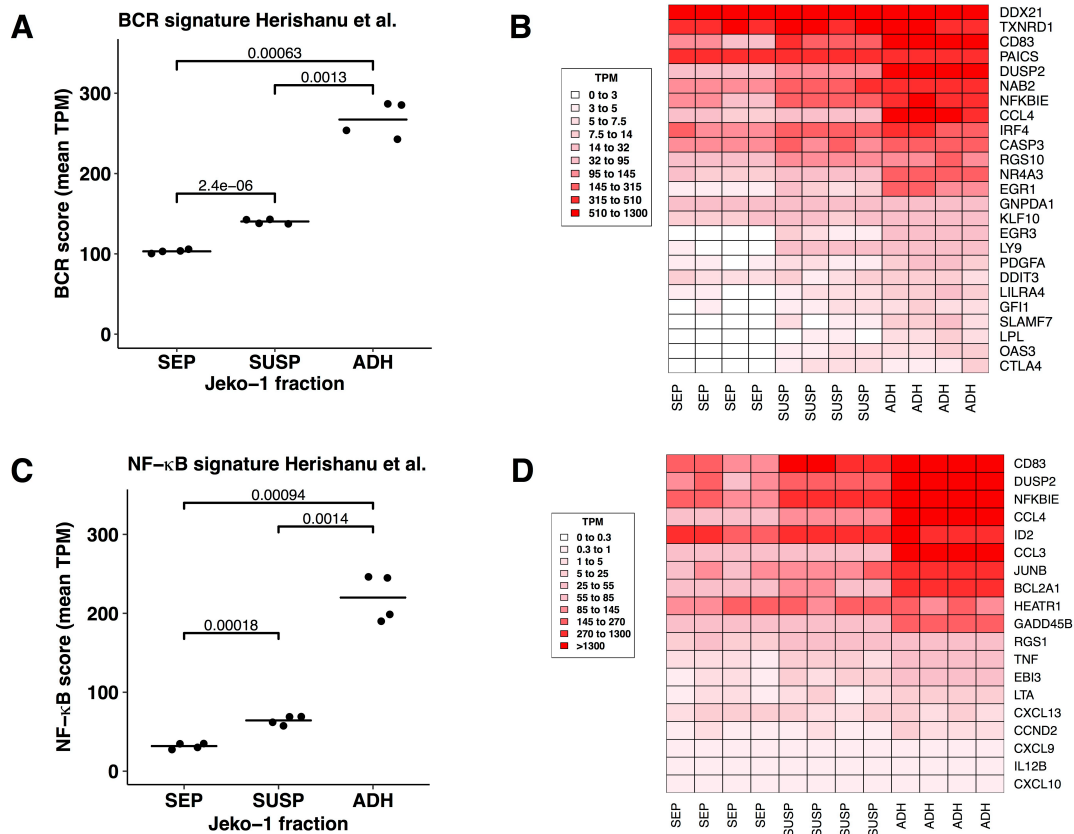
Supplementary Figure 2. Adhesion of Jeko-1 cells to MS-5 stromal cells leads to increased mRNA levels for B cell receptor and NF-κB signature genes.

B cell receptor signature and NF-κB signatures as described by Herishanu et al. [1]. SEP: mono-cultured Jeko-1 cells. SUSP: Suspension fraction of Jeko-1 cells co-cultured with MS-5 stromal cells. ADH: Jeko-1 cells adhered to MS-5 stromal cells in co-culture. A) Mean transcripts per million (TPM) expression level for all genes in the B cell receptor signature. The significance values indicated in the figure are student's t-test p-values for the indicated comparisons. B) Heatmap representation of the B cell receptor signature genes and their corresponding expression levels used in A. C) Mean TPM expression level for all genes in the NF-κB signature. The significance values indicated in the figure are student's t-test p-values for the indicated comparisons. D) Heatmap representation of the NF-κB signature genes and their corresponding expression levels used in C.

Supplementary Figure 3

Supplementary Figure 3. Visualization of STRING network analysis output.
Visualization of the STRING network analysis output for the unified list of differentially expressed genes and genes functionally classified by the gene set enrichment analysis (n=1458). Each point represents a gene and the edges in blue correspond to interactions based on "database" and "evidence" fields in the STRING interaction database.

Supplementary Figure 4
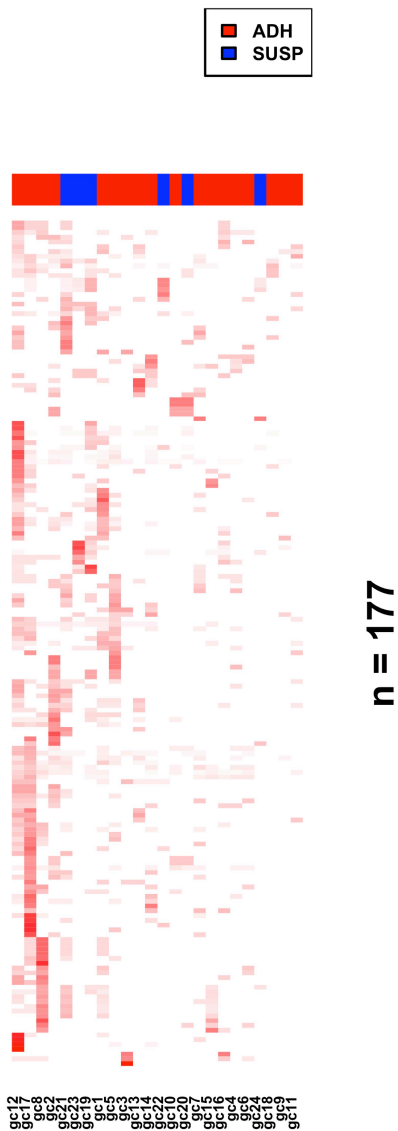
Supplementary Figure 4. Node genes and their connections to functional clusters

Visualization of interactions between 177 differentially expressed node genes not classified by the gene set enrichment analysis and their connections to genes in functional categories (c1-c24). Each row corresponds to a node gene and the intensity of red is proportional to the number of genes in each functional cluster that the particular node gene is connecting to. ADH: Genes with increased transcript levels in Jeko-1 cells adhered to MS-5 cells in co-culture. SUSP: Genes with decreased transcript levels in Jeko-1 cells adhered to MS-5 cells in co-culture.

SUPPLEMENTARY METHODS

Cell culture

All cells were cultivated in a humidified incubator at 37°C and 5% CO_2. The mouse stromal cell line MS-5[2] was purchased from DSMZ and maintained in αMEM-glutamax (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (H.I. FBS; Gibco), 2mM sodium pyruvate and 100U/mL penicillin and 100μg/mL streptomycin (Gibco)[2]. Stromal cells of murine rather than human origin were chosen to facilitate separation of RNAseq reads from stromal cells and adherent lymphoma cells (human) without the need for cell separation prior to RNA extraction, which has been shown to cause data artifacts[3, 4]. The adherence properties of murine stromal cells and their influence on human cancer cells are essentially indistinguishable from their human counterparts[5] but we cannot formally exclude species differences in the underlying gene-regulation. The mantle cell lymphoma cell line Jeko-1[6] was obtained from DSMZ and kept in RPMI-glutamax (Gibco) supplemented with 10% H.I. FBS and 100U/mL penicillin and 100μg/mL streptomycin until co-cultivation with MS-5.

Co-cultures were maintained in αMEM-glutamax supplemented with 10% H.I. FBS, 2mM sodium pyruvate and 100U/ml penicillin and 100μg/ml streptomycin. For global gene expression analysis, 5×10^5 MS-5 stromal cells were seeded 24h in advance to tissue culture treated 10cm dishes (TPP). Jeko-1 cells were subsequently added at a 10:1 ratio and after 24h a fraction of the input cells had adhered to the stromal cell layer while the majority remained in suspension. The suspension fraction was removed, adherent fraction—containing both Jeko-1 and MS-5 cells—washed once in PBS and the two fractions immediately subject to RNA extraction.

Cell-cell binding assay

6×10^4 MS-5 cells per well were seeded 24h in advance to 6 well plates (Corning). At 0h the established layer of adherent MS-5 cells was washed once in PBS followed by addition of 6×10^5 Jeko-1 cells per well to the 6-well plate. Following 24h co-culture, cells remaining in

suspension were removed, the adherent layer—consisting of MS-5 and Jeko-1 cells—was washed twice in PBS followed by addition of 6x10^5 CFDA SE labeled Jeko-1 cells per well. Jeko-1 cells were labeled with the cell permeable dye CFDA SE (Invitrogen) at a final concentration of 0.5μM in αMEM-glutamax, according to the manufacturers protocol.

RNA extraction, library preparation and sequencing

Total RNA was extracted using RNeasy (Qiagen) with QIAshredders (Qiagen) according to manufacturer instruction. Following quality assessment by TapeStation, libraries were prepared using TruSeq sample prep kit v2.0 (Illumina) according to the manufacturers protocol with mRNA purification step by poly-T oligo-attached magnetic beads. Samples were 16-plexed and sequenced using an Illumina HighSeq 2500 instrument generating a total of 230'700'000 2x101bp short reads (Table S1).

Species based read separation and mapping to reference genomes

Reference genomes hg19 (GRCh37) and mm10 (GRCm38) were obtained from UCSC. These were indexed using the species-based short read separation software Xenome (v1.0.1)[7] and the short read aligner Bowtie2 (v2.2.2)[8]. Raw paired end (PE) short reads were separated based on species origin using Xenome with k-mer length set to the default value of 25. During the course of this project a similar approach for species based read separation for xenograft breast cancer material has been reported elsewhere[9]. Separated, PE short reads of human and murine origin were aligned to reference genomes hg19 and mm10 respectively using the splice aware short read aligner Tophat2 (2.0.11)[10] with Bowtie2 (2.2.2) and Samtools (0.1.19)[11] using PE read input and default options. BAM files containing aligned reads were imported into R (3.1.1) and fragments per feature (concatenated exons for one gene) were counted using summarizeOverlaps from the Bioconductor (2.14) package GenomicAlignments (1.0.6) with counting mode set to "Union" and options singleEnd=FALSE, ignore.strand=TRUE, fragments=FALSE. Annotated genes were acquired through Bioconductor packages TxDb.Hsapiens.UCSC.hg19.knownGene   (v2.14.0)   and

TxDb.Mmusculus.UCSC.mm10.knownGene (v2.14.0). Transcripts per million (TPM) were calculated for mean read counts per gene for each cell fraction as previously described[12] using the concatenated exon lengths per gene for length of feature.

Differential gene expression analysis and GSEA

Differential gene expression between fractions of Jeko-1 and MS-5 cells was determined by normalizing raw read count tables and interrogating these for differentially expressed genes using the Bioconductor package DESeq (1.16.0)[13]. Normalized read count tables from DESeq were subsequently subject to gene set enrichment analysis (GSEA, Broad Institute, 2.2.0)[14, 15]. Canonical pathway gene sets (c2.cp.v5.0.symbols.gmt) and gene ontology gene sets representing biological processes (c5.bp.v5.0.symbols.gmt), available from the MSigDB (http://www.broadinstitute.org/gsea/msigdb/index.jsp) were included in the analysis[15]. Gene sets with fewer than 15 and more than 300 members were excluded from the analysis. Gene expression values were ranked by Signal2Noise and subject to 10000 permutations by shuffling of the gene sets. A leading edge analysis following the GSEA generated heatmaps with genes and functional annotations where clusters of genes (cluster criteria: $n_{genes} \geq 10$) could be annotated.

Microarray analysis

Two microarray datasets were downloaded from GEO[16]: GSE21029[1] and GSE70910[17]. Both studies utilized Affymetrix HG U133 Plus 2.0 arrays. Patient-matched samples from the peripheral blood and lymph node material were included in the analysis from GSE21029 (n=34) and when analyzing the GSE70910 dataset patient matched samples from peripheral blood and unsorted lymph node material were used (n=16). Bioconductor packages Affy and Limma were used for RMA normalization and differential expression analysis and the p-values were adjusted for multiple testing by false discovery rate. Overlaps between the previously published datasets and adhesion-related genes from the present study were interrogated by two-sided fisher exact tests. Given the bone-marrow origin of the MS-5

stromal cells it would be relevant to compare with clinical data comparing cells from bone marrow and peripheral blood. The CLL study[1] does include this comparison albeit with fewer samples and greater inter-sample variability than for the lymph node comparison. Tentative analysis of this data does suggest a similar trend in bone-marrow as for the lymph-node comparison but more data would be required to draw this conclusion.

Data Availability

The datasets generated and analyzed during the current study are available via the gene expression omnibus (GEO) repository[16] by accession number GSE99501.

Protein interaction network analysis

The unified list of differentially expressed genes (FDR q-value≤0.05) and genes in clusters (n=1458) were uploaded STRING interaction database[18] (v10) containing known interactions using "database" and "experimental evidence" as database criteria with the confidence level set to 0.4. A node gene was defined as having 8 or more interactions.

Cytokine levels in conditioned media

MS-5 stromal cells were seeded 4h in advance onto tissue culture treated 12w plates at $5x10^4$ cells per well. $25x10^4$ Jeko-1 cells were subsequently added to the stromal cells or to separate wells supplemented with anti-IgM at 1μg/ml and 10μg/ml (unlabeled goat F(ab')2 Anti-Human IgM, Southern Biotech). Mono cultured MS-5 and Jeko-1 cells were seeded as controls. At 24 and 48 hours cells were spun down and conditioned media was stored at -20 until further analysis. The samples were diluted 1:5 and analyzed by sandwich ELISA for human CCL3 and human CCL4 according to manufacturer's instructions (R&D Systems, DMB00 and DMA00).

A DEEPER PRESENTATION OF RESULTS

Differentially expressed genes and diffuse clusters *i-iv*, Figure 3A

Among the genes in the diffuse cluster groups *i-iv* are, among others, central players of the canonical and non-canonical NF-κB pathways such as *NFKB1* and *NFKB2* as well as the member of the Inhibition of Apoptosis (IAP) family, *BIRC3* (group *i*, Figure 3A), the latter is consistent with studies on DLBCL cell lines adhering to stromal cells[19]. Group *i* also contains components of the NF-κB pathway responsible for cytoplasmic retention of the NF-κB complex such as *NFKBIA*, *NFKBIE* and *NFKBIB* as well as anti-apoptotic genes like *CFLAR* (c-FLIP) and mediators of TNF-mediated activation of NF-κB such as *TRAF1*. This group also contained the cell-cell adhesion-related genes *ICAM1*, *ITGB2* (fold change 3.8 and 1.8, Group *i* in Figure 3A). Group *ii* includes *MALT1*, *CARD11* and *BCL10*, all activators of the NF-κB pathway (Figure 3A). Group *iii* contain the chemotactic molecules CCL3 and CCL4 that are known to be up-regulated upon BCR stimulation in malignant B cells[20]. The increases in transcript levels are also reflected at the protein level with co-culture induced induction levels of CCL3 and CCL4 similar to the levels that result from BCR stimulation by anti-IgM (Supplementary Figure 1). Notably CCL3 and CCL4, as well as the adhesion molecule ICAM1 and the central component of the NF-κB pathway NFKB1 are also members of the core gene signature identified by the present study. Increased transcript levels from anti-apoptotic genes may be due to co-culture dependent increased signaling through NF-κB[19, 21] which is up-regulated at the mRNA level in adherent MCL cells as indicated by cluster c1 and the grouping of genes denoted *i* in Figure 3. Similarly, the BCR regulated anti-apoptotic transcription factors *EGR1*, *EGR2* (Group *iv*, Figure 3A) also have elevated transcript levels in ADH cells[20, 22]. Another example suggesting increased signaling downstream of the BCR leading to immunomodulation is the increased expression of *CCL22*, which recruits regulatory T cells to malignant follicles in follicular lymphoma[23] (Table 1). In addition, we observed elevated transcript levels of the cytokine receptors CCR7 and CXCR5 (fold change

1.3 and 1.8 respectively) in the adherent MCL fraction, both of which are present in the leading edge but outside of the clusters.

Cell adhesion, migration and homing

The stromal cells express the chemotactic molecule CXCL12, known to be important for B cell migration and homing. Its receptor, CXCR4 had 2.1 fold lower transcript levels in the fraction of MCL cells that had adhered to the stromal cells when compared to those remaining in suspension. Lower cell-surface CXCR4 expression in MCL and CLL cells isolated from lymph nodes compared to peripheral blood has been described[1, 22]. This is consistent with the lower *CXCR4* mRNA levels in CLL cells residing in the bone marrow or lymph nodes as compared to those in circulation[24]. It appears that reduced transcription of *CXCR4* accounts for at least part of the reduction in cell surface CXCR4 protein levels in microenvironment associated cells, even though increased turn-over rates of internalized CXCR4 protein could also contribute, as proposed previously[25]. Significantly, inhibition of CXCR4 by the small molecule AMD3100 disrupts interactions between multiple myeloma cells and stromal cells, making the malignant cells more susceptible to therapy[26].

Another example of increased cytokine gene expression in the adherent MCL cells is CCR7. CCR7 is, together with CXCR4, overexpressed on the surface of CLL cells from patients with a more advance disease progression[27] and treatment of MCL xenograft mice with a monoclonal anti-CCR7 antibody delayed tumor appearance, significantly reduced tumor volume and attenuated dissemination to distant lymphoid and non-lymphoid organs[28]. CCR7 is often expressed by regulatory T-cells and dendritic cells and plays a central role for their homing and migration towards CCL19/CCL21 gradients[29]. Expression of the genes encoding these ligands was however not detected in this study and therefore the CCR7 may not play a functional role in this system due to the absence of microenvironmental niche cells expressing appropriate ligands in the present model system.

Other examples of interest are the immunomodulatory chemokines, CSF1 and NAMPT, which have higher transcript levels in adherent MCL cells and which promote differentiation and polarization of monocytes into immunosuppressive M2 macrophages[30, 31].

Cell proliferation

The number of differentially expressed genes and enriched gene sets for genes with lower transcript levels in the adherent co-culture fraction were fewer than for up-regulated genes. Notably, most were related to the cell cycle and in particular to early steps in mitosis such as pro-metaphase, metaphase as well as formation of centrosomes and kinetochores and include genes such as *AURKA, CCNB2, PLK1, BUB1, CENPE, KIF20A* and *KIF5A*. Adhesion-mediated alterations to cell cycle progression are further supported by a 1.4 fold decrease in transcript levels of the proliferation marker Ki67 in the ADH fraction along with a 1.5 fold increase in transcript level for the cell cycle inhibitor p21^{Cip1}.

Protein interaction network analysis

299 of the 1050 differentially expressed genes between ADH and SUSP (~30%) were functionally classified by the GSEA analysis. The 24 clusters contain 603 of the 813 genes in the GSEA leading edge and 191 of these genes had significantly altered transcript levels. Thus a majority of differentially expressed transcripts did not contribute to the GSEA functional classification and therefore there remained a formal possibility that this set of unclassified genes might represent processes and pathways not identified by the GSEA analysis.

To address this we used protein-interaction network analysis to determine the extent to which protein interactions couple the set of unclassified genes to the gene clusters identified by GSEA. The unified list of differentially expressed genes (FDR q-value≤0.05) and genes in clusters (n=1458) was analysed using the STRING interaction database[18] (v10) containing known interactions using "database" and "experimental evidence" as database criteria with the confidence level set to 0.4. The resulting interaction network (Supplementary Figure 3)

connects 1085 of the 1458 analyzed genes to each other. Node genes were subsequently defined as those having 8 or more interactions, as has been described previously[32]. 510 genes were classified as "node genes", defined as nodes connecting to eight or more other genes within the dataset. Each of the 510 networks defined by these node genes contains one or more genes that are also a member of one or more of the 24 GSEA clusters (c1-c24), indicating a strong overlap between the main component networks making up the interaction network and the functional categories identified by GSEA analysis. 177 of the 510 component networks making up the STRING-generated interaction network have node genes that lie outside the 24 clusters defined by GSEA analysis. Supplementary Figure 4 shows how this set of 177 component networks connects to the 24 GSEA clusters, with regard to both the number of clusters that interact with each component networks (red lines) and the proportion of genes in each component network that interact with each cluster (intensity of red lines). It is clear that most component networks overlap with multiple clusters and that overlapping genes tend to be concentrated to a subset of clusters (left side of Supplementary Figure 4). The 510 component networks contain 495 adhesion-regulated genes in addition to those in GSEA clusters and together these groups contain about 65% of the adhesion regulated genes. The results do not provide support for the existence of important functional categories of adhesion-associated genes over and above those identified by GSEA analysis. A complete table with node proteins and their corresponding interactions to the 24 functional clusters is presented in Supplementary Table 10.

SUPPLEMENTARY TABLES

Note on Tables: Supplementary Table 1 contains read counts, Supplementary Table 2-10 contain lists of differentially expressed genes and enriched gene sets. As the tables are large they can be downloaded separately as SupplementaryTables_S1-S10.xlsx where the supplementary tables are provided as separate sheets. Legends to all supplementary tables are presented below.

Supplementary Table 1

S1_RAW_RNAseq_ReadNumbers.xlsx
RNA sequencing workflow and read numbers

Paired end read counts for the RNA sequencing data processing steps, per sample before and after species based read separation. The columns are: ID: unique sample id, Sample description, reads in: millions of paired end reads before species based read separation, hg19: number of paired end reads classified as human at species based read separation by Xenome, mm10: number of paired end reads classified as mouse at species based read separation by Xenome.

Supplementary Table 2

S2_Jeko1_ADH-SUSP_FDR0.05
List of differentially expressed genes between co-cultured Jeko-1 cells and Jeko-1 cells adhered to MS-5 stromal cells.

1050 differentially expressed genes (FDR q-value ≤ 0.05) between adherent and suspension MCL cells in co-culture with MS-5 stromal cells as determined by DESeq. Sorted by the adjusted p-value. name: official gene symbol, entrezgene: entrez gene id, SEP: mono-cultured Jeko-1 cells, SUSP: Jeko-1 cells in suspension in co-culture with MS-5 cells, ADH: Jeko-1 cells adhered to MS-5 cells in co-culture, TPM; transcripts per million, FC; fold change from DESeq, pval and padj: p-value and respective adjusted p-value from DESeq output.

Supplementary Table 3

S3_Jeko1_ADH-SEP_FDR0.05
List of differentially expressed genes between mono-cultured Jeko-1 cells and Jeko-1 cells adhered to MS-5 stromal cells in co-culture.

3453 differentially expressed genes (FDR q-value ≤ 0.05) between adherent MCL cells in co-culture with MS-5 stromal cells and mono-cultured MCL cells as determined by DESeq, sorted by the adjusted p-value. name: official gene symbol, entrezgene: entrez gene id, SEP: mono-cultured Jeko-1 cells, SUSP: Jeko-1 cells in suspension in co-culture with MS-5 cells, ADH: Jeko-1 cells adhered to MS-5 cells in co-culture, TPM: transcripts per million, FC; fold change from DESeq, pval and padj: p-value and respective adjusted p-value from DESeq output.

Supplementary Table 4

S4_Jeko1_SUSP-SEP_FDR0.05
List of differentially expressed genes between mono-cultured Jeko-1 cells and co-cultured Jeko-1 cells in suspension.

1471 differentially expressed genes (FDR q-value ≤ 0.05) between suspension MCL cells in co-culture with MS-5 stromal cells and mono-cultured MCL cells as determined by DESeq, sorted by the adjusted p-value. name: official gene symbol, entrezgene: entrez gene id, SEP: mono-cultured Jeko-1 cells, SUSP: Jeko-1 cells in suspension in co-culture with MS-5 cells, ADH: Jeko-1 cells adhered to MS-5 cells in co-culture, TPM: transcripts per million, FC; fold change from DESeq, pval and padj: p-value and respective adjusted p-value from DESeq output.

Supplementary Table 5

S5_MS5_COCULT-SEP_FDR0.05
List of differentially expressed genes between mono-cultured MS-5 cells and co-cultured MS-5 cells.

100 differentially expressed genes (FDR q-value ≤ 0.05) between MS-5 stromal cells co-cultured with MCL cells and MS-5 stromal cells in mono-culture as determined by DESeq, sorted by the adjusted p-value. name: official gene symbol, entrezgene: entrez gene id, SEP:

mono-cultured Jeko-1 cells, SUSP: Jeko-1 cells in suspension in co-culture with MS-5 cells, ADH: Jeko-1 cells adhered to MS-5 cells in co-culture, TPM: transcripts per million, FC; fold change from DEseq, pval and padj: p-value and respective adjusted p-value from DEseq output.

Supplementary Table 6

S6_GSEA_CP_GObp_Jeko-1_ADH
GSEA output table ADH.

Enriched gene sets for genes with higher transcript levels in the adherent Jeko-1 cell fraction in co-culture with MS-5 stromal cells.

Supplementary Table 7

S7_GSEA_CP_GObp_Jeko-1_SUSP
GSEA output table SUSP.

Enriched gene sets for genes with lower transcript levels in the adherent Jeko-1 cell fraction in co-culture with MS-5 stromal cells.

Supplementary Table 8

S8_GSEA_leading_edge_ADH
GSEA leading edge output table.

GSEA leading edge output table for genes with increased transcript levels in the MCL cell fraction adhered to MS-5 stromal cells in co-culture. Columns represent 653 genes and rows 182 functional groups. The number 1 in the table is indicative of the presence of a gene in a particular functional group. The row denoted "total" is the sum of functional categories within the leading edge in which the particular gene is represented. "inclust" indicates whether the gene is included in a functional cluster category and "FC" is the transcript level fold change for differentially expressed genes in adherent MCL cells in co-culture relative to suspension MCL cells in co-culture with MS-5 stromal cells.

Supplementary Table 9

S9_GSEA_leading_edge_SUSP
GSEA leading edge output table.

GSEA leading edge output table for genes with decreased transcript levels in the MCL cell fraction adhered to MS-5 stromal cells in co-culture. Columns represent 166 genes and the rows represent 20 functional groups. 1 in the table is indicative of a particular genes presence in a particular functional group. The row denoted "total" is the sum of functional categories within the leading edge in which the particular gene is represented. "inclust" indicates whether the gene is included in a functional cluster category and "FC" is the transcript level fold change for differentially expressed genes in adherent MCL cells in co-culture relative to suspension MCL cells in co-culture with MS-5 stromal cells.

Supplementary Table 10

S10_node_connection_DEclust
Table of "node genes" derived from STRING interaction analysis and their connections to the 24 functional clusters characterized by GSEA.

510 genes classified as node proteins for having 8 connections or more to genes that either had significant changes in transcript levels or that were present in the leading edge from GSEA, where the connections were derived from the STRING database. Each row represents a central node gene and the number of connections for that particular gene to differentially expressed genes not in present in the leading edge analysis (DE), differentially expressed genes present in the leading edge output but not belonging to a functional cluster (DELE) and genes in the functional clusters c1-c24 from the leading edge analysis output. "sum" is the total number of connections for the central node protein within the dataset and "nodelocation" indicates where in the dataset (DE, DELE or c1-c24) the node gene is present. If the node gene presents with significant differential mRNA levels between the adherent and suspension MCL fractions in co-culture the $\log_2$ (fold change) is presented in the column "log2FC".

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