Implication of clusterin in TNF-α response of rheumatoid synovitis: lesson from in vitro knock-down of clusterin in human synovial fibroblast cells

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RHEUMATOID ARTHRITIS (RA) is a chronic inflammatory disease established December 13, 2011; doi:10.1152/physiolgenomics.00095.2010.— Recently clusterin (CLU) was reported to be an inhibitor of NF-κB pathway and involved in rheumatoid arthritis (RA) synovitis. This study was designed to decipher the molecular network linked to CLU expression in FLS (fibroblast-like synoviocytes) and evaluate the consequences of its low expression in conditions of TNF-α stimulation. FLS were transfected with siRNA for CLU or not and cultured for 24 and 48 h with TNF-α or not. Pan-genomic gene expression was assayed by DNA microarray. The gene network around CLU and gene interactions were analyzed with the Ingenuity Pathway Analysis software. Downregulation of CLU resulted in modification of the expression of genes known to be directly linked to CLU and for almost 5% of the tested genes (857 out of 17,225); the upregulation of a small group of gene (e.g., TIAM1) emphasizes the hypothetical role of CLU in the pseudotumoral characteristic of FLS. The comparison of gene expression with or without TNF stimulation allowed the classification of sampled with good concordance. Moreover, differential comparison showed that CLU downregulation in RA led to a profound modification of the TNF-α response of rheumatoid arthritis; fibroblast-like synoviocytes; microarray; siRNA; tumor necrosis factor

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FLS cultures. We obtained synovial tissue (using methods previously described) from patients with RA who fulfilled the RA criteria of the American College of Rheumatology (1) and had given their full consent. In brief, synovial tissues collected through remedial synovectomy or arthroplasty were finely minced and treated with 4 mg/ml collagenase-dispase (Sigma-Aldrich) for 2 h at 37°C (see Ref. 5). FLS were allowed to adhere to tissue culture plates, and nonadherent cells were removed. FLS were grown to confluence (70%) in culture flasks containing complete medium RPMI 1640 supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, and 500 units/ml of penicillin. All experiments were performed using FLS from the third passage. At this time, there were <2% contaminating lymphocytes, natural killer cells, and macrophages. TNF-α stimulation was achieved by adding 20 ng/ml of TNF-α to the medium culture for 24 or 48 h.

RNA preparation. Total RNA was extracted from cultured cells in RLT RNA extraction buffer (Qiagen, Rneasy kit), and treated with DNase I to eliminate genomic DNA contamination. The integrity and purity of the total RNA, and of the cRNA, were analyzed twice using
a Bioanalyzer 2100 and RNA kit 6000 LabChip (Agilent Technologies). Only total RNA with a 28S/18S ratio >1.7 was used. RNA concentrations were measured using NanoDrop (NanoDrop Technologies).

cRNA synthesis and probe array hybridization and gene expressions. Microarray experiments were performed on Illumina Human RefSeq-8 Whole Genome BeadChip (a genome-wide array with 24,350 probes). Data are available at Array Express (http://www.ebi.ac.uk/arrayexpress, accession ID: E-TABM-653). Fluorescence data were processed with GeneSpring (Agilent Technologies, Santa Clara, CA) and R Bioconductor. Gene expression levels were calculated using the Illumina BeadStudio software. Normalized gene expression values were produced using GeneSpring’s per-gene normalization. We used P value as a threshold for gene selection. Only probes showing data with detection rate ≥0.95 were selected for analysis. When adding a criterion of differential level of expression at 1.5, we obtained a second shorter list of gene (gene lists are available upon request). The group comparisons were done using Student’s t-test. To estimate the false discovery rate (FDR) we filtered the resulting P values at 5% and used the Benjamini and Hochberg FDR correction. Bonferroni FDR correction, or no correction. Ratios were then classified according to their relative distance to the mean in the ratio population, expressed in fold of standard deviation, and filtered at threshold 2× SD.

Cluster analysis was performed by hierarchical clustering in GeneSpring using the Spearman correlation similarity measure and the average linkage algorithm.

Data were subsequently submitted to Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Redwood City, CA) to model relationships among genes and proteins and to construct putative pathways and relevant biological processes. Pathways were generated by placing the genes that were expressed with a significant statistical difference and already known into IPA. Each Illumina gene identifier was mapped to its corresponding gene object in the Ingenuity knowledge base. Networks of the genes were then algorithmically generated based on their “connectivity” derived from known interactions between products of these genes. Fisher’s exact test was used to estimate pathways enrichment in our list of significant genes. Pathways classification is achieved by statistical validity [−log(P value) reckoning].

Kmeans was computed with 100 interactions, and Pearson correlation was used for similarity measures. Once again, functional analysis were performed with Ingenuity software using the Fisher exact test to evaluate significant results filtered by cutoff of 0.05. The two-way ANOVA was performed with Partek Genomics suite (Partek, St. Louis, MO) following the method of the moments considering CLU effect, TNF effect, and CLU*TNF effect. The formula used in the model is Yi,j,k = \mu + CLUi + TNFj + CLU * TNFij + eijk, where Yi,j,k represents the kth observation on the ith CLU and jth TNF; \mu is the common effect for the whole experiment; eijk represents the random error present in the kth observation on the ith CLU and jth TNF. The following contrast(s) (15a) was performed to compare: CLU+ * TNF+ vs. CLU− * TNF−.

Small interfering RNA transfection. Small interfering RNA (siRNA) transfection of FLS (1–5) (see Ref. 4) were performed in third-passage synoviocytes by using Lipofectamine 2000 (Invitrogen Life Technologies). Cells were seeded at 50% confluence 2 days before siRNA transfection in six-well plates containing 4 ml of complete medium. The day before transfection, the cells were cultured in complete medium without antibiotics. Preliminary experiments using FITC-labeled siRNA allowed us to determine that the mixture of 5 μl of Lipofectamine and 150 pM CLU siRNA is sufficient for a transfection efficiency of 90–100% during 48 h. Cells were cultured for 1 h before transfection in 1.5 ml of Opti-MEM without serum and antibiotics and then treated with the siRNA complexes. Vitality of cells was good as cells did not show apoptotic phenotype. Five hours after starting the incubation, we added 2 ml of complete medium to the culture. Transfection was assayed after 24–72 h. C1 sense, 5'-cCrArGrArCrCrUrUrCrArCTT-3'; C1 antisense 5'-rgrUr-gCrGrGrArCrGrAuUrgrrggrArCTT-3'; control scrambled sense, 5'-rgrUr-CUrGCrArCrGrUrArCrATT-3'; control scrambled antisense, 5'-UrgUrArCrArCrUrGrUrgrgrArArGrCTT-3'. C1 linkage to CLU gene has been described previously (4, 17).

Quantitative RT-PCR. A total of 100 ng of cRNA used for the microarray analyses was reverse-transcribed to cDNA, and the resulting cDNA was amplified. In brief, the first-strand cDNA was synthesized (from 100 ng of cRNA) using random primers and SuperScript-II retrotranscriptase (200 U/μl; Invitrogen). For quantitative RT-PCR Taq Man method was performed as previously described (1a). We used primer sequences (invented by Applied Biosystems, Courtaboeuf, France). For IL-8 pathway, we used IRAK3: Hs00936103_m1; and prostaglandin-endoperoxide synthase-2 (PTGS2, Hs00153133_m1). For Wnt pathway we used: AKT1: Hs00260717_m1; PPP2R4: Hs00603515_m1; vascular cell adhesion molecule-1 (VCAM1, Hs00365486_m1). Hypoxanthine guanine phosphoribosyltransferase (HPRT, Hs99999909_m1) was used as housekeeping control. All PCRs were performed in triplicate. Relative mRNA levels for each sample were quantified using the Ct approach (fluorescence threshold), normalized with respect to HPRT RNA as the standard.

RESULTS

Gene profile of FLS following transfection of siRNA for CLU. Treatment of FLS cultures with CLU siRNA induced knock-down of both CLU mRNA and CLU protein (4). In these experiments, we transfected FLS of RA patient with either siRNA for CLU (siCLU) or scrambled oligos and cul-
tured them for 24 h before a pan-genomic analysis with the bead array system of Illumina: 24,350 probes (among them 4,350 are unmapped according to Ingenuity) resulting in 17,225 genes. The comparison of the 857 significantly modified genes (Supplemental Data S1) between siCLU (S1–S5) transfected FLS and scrambled (C1–C5) oligos transfected FLS allows a good ranking of classification, as represented in Fig. 1A. This technique can assay for the direct implication of CLU inhibition. Nevertheless, expression factors for differentially expressed genes were generally <2.

We then selected genes directly linked to CLU and modified with a statistical significance because of CLU inhibition. As represented in Fig. 1B, among the group of seven genes directly linked to CLU, two were downregulated by PDPK1, a phosphokinease and SORBS3. Five genes were slightly but significantly upregulated from 1.15- to 1.63-fold, ranking SLC9A8, TIAM1, KIAA1409, CDH12 encoding for cadherin, CTSL1 encoding for cathepsin L1.

Downregulation of CLU induced a drastic change of FLS-response to TNF-α. The analysis of genes expressed following TNF-α stimulation in the presence of CLU inhibition allowed us to select 853 and 840 genes in siCLU transfected FLS and controls, respectively. As represented in Fig. 2A (siCLU transfected FLS) and 2B (scrambled oligo FLS), the hierarchical analysis of gene expression allows a classification that is linked to the stimulation used, with or without TNF-α. When we performed a nonsupervised analysis with all the samples, we observed that TNF-α stimulation masked CLU impact (Supplemental Data S2). Therefore we performed a supervised analysis allowing us to analyze CLU’s impact in the context of TNF-α stimulation or no TNF-α stimulation. Indeed, this model allowed a quite clear comparison between three different pathophysiological states of what could be considered as synovial stimulation in RA (Fig. 2C). The comparison between siCLU transfected FLS and scrambled control oligos transfected FLS allowed us to build three lists of genes as represented in Fig. 2C: 497 genes modulated mainly by siCLU transfection and TNF only (red), 356 genes modified due to TNF stimulation in siCLU FLS and control FLS (yellow), and 484 modulated genes in control FLS after TNF stimulation (green).

1 The online version of this article contains supplemental material.

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Fig. 2. CLU knock-down modifies TNF-α response of FLS. We compared 5 samples of rheumatoid arthritis (RA) FLS transfected with siRNA for CLU (siCLU) (S1–S5) with 2 siCLU transfected FLS stimulated with TNF for 24 h (TNF1 and TNF2). Five samples of RA FLS transfected with scrambled (C1–C5; A) were compared with scrambled transfected FLS stimulated with TNF-α for 24 h (TNF3 and TNF4; B). C: Venn diagram built with 497 genes modulated by siCLU transfection and TNF only (red), 356 genes modified due to TNF stimulation in siCLU FLS and control FLS (yellow), and 484 modulated genes in control FLS after TNF stimulation (green).
differentially expressed following TNF-α stimulation after CLU knock-down, differentially expressed genes were analyzed in silico using IPA software (Ingenuity). For each condition, we observed multiple interconnected networks between gene products. Within networks, certain gene products had numerous known interactions with other products (high connectivity), while other products had relatively few interactions.

Histogram representation of the main relevant biological functions, represented for main biological pathway families, is substantially modified in the presence of CLU inhibition (Fig. 3). Indeed, many features are common between the pathways obtained from the list of the 356 genes modified with TNF stimulation regardless of CLU expression and the 484 modulated genes in FLS during TNF stimulation in the presence of CLU expression. From the list of the 497 genes modulated mainly by siCLU transfection in the presence of TNF stimulation, three new main families appeared, namely IL-8 signaling and Wnt-β catenin signaling, and the tight junction signaling genes. For both IL-8 and wnt pathways, we followed expression of two differentially expressed genes by quantitative real-time PCR: IRAK3 and PTGS2 for the IL-8 pathway and AKT1 and VCAM1 for the Wnt pathway. Regarding the Wnt pathway, we observed a ratio of expression of 2.2 and 1.8 for AKT1 and VCAM-1, respectively, when we compared TNF-α stimulation in the absence vs. presence of CLU. On the other hand, IRAK3 and PTGS2 expression was found to be upregulated by 1.6- and 2.6-fold, respectively, in the same conditions. All these results were in accordance with results obtained in microarray experiment.

What is noteworthy is that by using a different kind of analysis, Kmeans, we could establish different kinetics of gene modification in our system of modulation (with or without TNF, with or without CLU), which is represented in Fig. 4. Group 1 (Fig. 4A) and group 3 (Fig. 4B) are genes mainly regulated by TNF regardless of CLU expression. Besides these genes connected to TNF modifications, we confirmed the raising of two sets of genes under CLU dependence (cluster 2 and 4, respectively; Fig. 4, B and D). Performing functional and canonical pathway analysis of these sets of genes, we could once again confirm the modification of genes implicated in inflammation, among them the wnt-β catenin signaling genes. We then performed a global analysis using an unsupervised ANOVA calculation followed by a two-way ANOVA on all data to test the validity of the model. We confirmed that the main factor of gene modulation was TNF (50% of the total gene variations), whereas the CLU effect was minor (2% of the total gene variations). The two-way ANOVA allowed us to differentiate the effects of CLU, TNF, or CLU + TNF on gene expression. The contrasts calculation allowed the assessment of the real impact of CLU on the TNF response leading to the identification of a molecular signature made of 232 genes (P value 0.01, see supplemental data). A

![Fig. 3. Histogram of the 10 main relevant biological pathways modulated by TNF-α stimulation in FLS in either the presence or absence of CLU knock-down.](image-url)
Fig. 4. Analysis of genes modulated by CLU using Kmeans. Expression profiles were analyzed by Kmeans and clustered into 4 groups (A, B, C, D). Clusters 2 (B; 708 probes) and 4 (D; 664 probes) were selected according to their relevant profile expression shapes. Functional analysis on biological functions (E, F) or canonical pathways (G, H) was performed on the selected Kmeans clusters.
selected set of the most significantly differentially expressed genes is shown in Table 1.

**DISCUSSION**

As we previously showed, transfection of FLS in RA patients with siCLU induced a major inhibition of CLU expression in terms of RNA and protein expression. Because we also showed that CLU was downregulated in RA (4), siCLU FLS could be used to test TNF-α stimulation’s implications in the context of CLU hypoxpression. Illumina chip arrays allow a broad analysis and ranking of genes and are therefore considered to be an appropriate tool for the study of pan-genomic analysis in our system. Quantification of gene expression in the presence of TNF-α was then the next step in assaying for synovitis stress during RA. Statistical analysis identified several sets of genes, among them were those that are directly linked to TNF stimulation (356 regardless of CLU inhibition) and those that could be related to CLU inhibition (497 in the presence of CLU inhibition and TNF). This sheds light on consistent modification of FLS gene expression after CLU inhibition, because 400 genes were differently expressed. This system allowed us to explore the network around CLU, which is still poorly characterized. CLU silencing has been shown to be associated with increased apoptosis and finally sensitization to genotoxic stress (17). We demonstrate that increasing CLU expression in RA FLS induces apoptosis of transfected cells. Our present work illustrates the potential involvement of CLU downregulation in FLS pseudotumoral phenotype in RA. Indeed, TIAM1, a gene known to be involved in tumor progression, is overexpressed in siCLU-induced lower expression of the inhibitor of NF-κB (NFκBIB) and tight junction signaling, and cadherin 11 participates in the formation of layers in vitro; moreover, synovial cadherin-11 determines the behavior of synovial cells in their proinflammatory and destructive tissue response in inflammatory arthritis (9). It is not known if cadherin 12 also plays a role in this process.

The main original result of our work is the model we propose to analyze CLU impact on FLS, confirmed by two methods of analysis. This is further evidence that CLU inhibition considerably modifies genes expression of TNF-α stimulated FLS. By the means of an IPA system using the list of these affected genes (497), we determined three main signaling pathways, namely IL-8 signaling, Wnt/β-catenin, and tight junction signaling, which are all implicated in RA synovitis (4, 11, 13). According to our in-depth analysis of the CLU impact on TNF response, genes implicated in protein or nucleotide synthesis and regulation (WDFD3, PPIC, DNAJC30, ATP6V1C1, ERAP1) are upregulated in the absence of CLU. In line with the inhibitory function of CLU on NF-κB, we observed that downregulation of CLU induced lower expression of the inhibitor of NF-κB (NFκBIB) as well as a upregulation of IL-33. These results

Table 1. Genes affected by CLU during TNF stimulation

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Entrez Gene Name</th>
<th>Location</th>
<th>Type(s)</th>
<th>P Value</th>
<th>Fold Change</th>
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<tr>
<td>WFD3</td>
<td>WAP four-disulfide core domain 3</td>
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<td>other</td>
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<td>IBSP</td>
<td>integrin-binding sialoprotein</td>
<td>extracellular space</td>
<td>kinase</td>
<td>1.12E-03</td>
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<tr>
<td>BMPR1A</td>
<td>bone morphogenetic protein receptor, type IA</td>
<td>plasma membrane</td>
<td>kinase</td>
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<td>GK</td>
<td>glycerol kinase</td>
<td>cytoplasm</td>
<td>kinase</td>
<td>2.89E-04</td>
<td>1.4</td>
</tr>
<tr>
<td>CCDC117</td>
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<td>unknown</td>
<td>other</td>
<td>9.07E-04</td>
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</tr>
<tr>
<td>PPIC</td>
<td>peptidylprolyl isomerase C (cyclophilin C)</td>
<td>cytoplasm</td>
<td>enzyme</td>
<td>5.98E-04</td>
<td>1.3</td>
</tr>
<tr>
<td>DNAJC30</td>
<td>Dna (Hsp40) homolog, subfamily C, member 30</td>
<td>cytoplasm</td>
<td>other</td>
<td>1.31E-03</td>
<td>1.3</td>
</tr>
<tr>
<td>ATP6V1C1</td>
<td>ATPase, H^+ transporting, lysosomal 42 kDa, V1 subunit C1</td>
<td>cytoplasm</td>
<td>transporter</td>
<td>6.25E-07</td>
<td>1.3</td>
</tr>
<tr>
<td>ERAP1</td>
<td>endoplasmic reticulum aminopeptidase 1</td>
<td>extracellular space</td>
<td>peptidase</td>
<td>1.37E-03</td>
<td>1.3</td>
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<tr>
<td>IL33</td>
<td>Interleukin-33 (cytokine)</td>
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<td>cytokine</td>
<td>2.83E-03</td>
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<td>FGFI3</td>
<td>fibroblast growth factor 13</td>
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<td>growth factor</td>
<td>5.3E-03</td>
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<tr>
<td>HRK</td>
<td>harakiri, BCL2 interacting protein (contains only BH3 domain)</td>
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<td>1.2</td>
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<td>other</td>
<td>6.97E-04</td>
<td>1.2</td>
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<tr>
<td>BAD</td>
<td>Bcl2-associated agonist of cell death</td>
<td>cytoplasm</td>
<td>death regulator</td>
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<td>1.4</td>
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<td>enzyme</td>
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<td>other</td>
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<tr>
<td>ATP6V0E2</td>
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<td>cytoplasm</td>
<td>enzyme</td>
<td>8.60E-04</td>
<td>1.3</td>
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<td>unknown</td>
<td>other</td>
<td>7.96E-05</td>
<td>1.3</td>
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<tr>
<td>NFkBIB</td>
<td>nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, beta</td>
<td>nucleus</td>
<td>transcription regulator</td>
<td>2.1E-03</td>
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<td>HIC1</td>
<td>hypermyelinated in cancer 1</td>
<td>nucleus</td>
<td>transcription regulator</td>
<td>8.31E-04</td>
<td>1.4</td>
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<td>cytoplasm</td>
<td>kinase</td>
<td>4.40E-05</td>
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<td>small nuclear RNA activating complex, polypeptide 2, 45 kDa</td>
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<td>transcription regulator</td>
<td>4.29E-05</td>
<td>1.5</td>
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<td>other</td>
<td>6.44E-04</td>
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<td>ARHGDA</td>
<td>Rho GDP dissociation inhibitor (GDI) alpha</td>
<td>cytoplasm</td>
<td>other</td>
<td>6.24E-04</td>
<td>1.6</td>
</tr>
</tbody>
</table>

A list of 232 genes was built calculating the P value of gene modification (P < 0.01) for the contrast condition (TNF+ * CLU− vs. TNF+ * CLU+). We selected the highest P values to present a list of gene with variation due to CLU: upregulated (positive fold change), and downregualted (negative fold change).
argue for a significant inflammatory action of CLU in RA synovitis.

**Conclusion**

DNA Chips can assay for RA FLS gene expression after CLU inhibition under TNF-α pressure. Our study shows that CLU inhibition results in the modulation of the expression of genes linked in networks that are known to be linked to RA. These results argue that CLU downregulation in FLS participate in the aggressiveness of the FLS.

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**AUTHOR CONTRIBUTIONS**


**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

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