Microarray profiling of L1-overexpressing endothelial cells reveals STAT3 activation via IL-6/IL-6Rα axis

Elena Magrini, Ugo Cavallaro, Fabrizio Bianchi

Department of Experimental Oncology, European Institute of Oncology, Milan, Italy
Molecular Medicine Program, Department of Experimental Oncology, European Institute of Oncology, Milan, Italy

A B S T R A C T

We recently identified a novel role for the L1 transmembrane glycoprotein (also known as L1CAM or CD171) in the regulation of tumor angiogenesis and vessels stabilization. L1 overexpression in cultured endothelial cells of the lung (luECs) exerted a pleiotropic effect in that it regulated proliferation, migration, tubulogenesis, vascular permeability, and endothelial-to-mesenchymal transition (EndMT). In addition, we provided strong evidence that antibody-mediated targeting of L1 may be an effective strategy for vessel normalization with the potential to increase efficacy of chemotherapeutic agents.

High-throughput microarray expression profile revealed that L1 modulates the expression of hundreds of genes mainly involved in cell cycle regulation, DNA replication, cellular assembly, migration, development and organization. By using a ‘pathway-oriented’ analysis strategy we were able to identify a network of 105 genes modulated by L1 through the predicted activation of five transcription factors: STAT1, STAT2, STAT3, IRF7, and ATF4. Indeed, L1 overexpression resulted in the strong induction of STAT3 phosphorylation which was abolished by antibody-mediated neutralization of IL-6Rα. These results indicated that L1 promoted STAT3 activation via the IL-6/IL-6Rα axis.

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Experimental design, materials and methods

Cell culture

Mouse luECs were immortalized with polyoma middle T antigen and cultured in MCDB131 medium (Gibco; Life Technologies) supplemented with 20% FBS (Invitrogen), 2 mM l-glutamine (Lonza), 1 mM Na-pyruvate (Gibco; Life Technologies), 100 μg/ml heparin (Sigma-Aldrich), and 50 μg/ml EC growth supplement (ECGS) obtained from calf brain. ECs were seeded on plates coated with glutaraldehyde-crosslinked gelatin and cultured in complete medium for 4 days to reach confluence. A complete description of cell culture and transfection conditions can be found in [1].

RNA preparation

Total RNA was extracted from ~5 × 10⁶ cells using the RNeasy Mini Kit (Qiagen) following manufacturer’s instruction. Quality control of the RNA samples was performed using an Agilent Bioanalyzer 2100 (Agilent Technologies). The RNA from three independent extractions was processed for each of the cell line under analysis.
Gene expression profiling

A total of 150 ng of RNA from each sample was used for RNA quality check, labeling and hybridization on a Mouse Gene 1.0 ST Genechip array according to the manufacturer’s instructions (Affymetrix). Three independent biological replicates were performed for each condition (L1-transfected vs. mock-transfected luECs). Data were pre-processed with AGCC (Affymetrix) and Expression Console 1.1. We used the robust multi-array average (RMA) [2] to normalized data. A total of 35,512 probesets were loaded and 819,041 PM probes were used for analysis. The library file “MoGene-1.0-st-v1.CDF” was used.

Next, we uploaded normalized data in BRB ArrayTools (http://linus.nci.nih.gov/BRB-ArrayTools.html) to run SAM analysis (http://www-stat.stanford.edu/~tibs/SAM/) in order to identify differentially expressed genes upon L1 overexpression in luEC cells. The SAM parameters of analysis were the following:

- Number of classes: 2
- Number of probesets: 35,512
- Target proportion of false discoveries (q-value): 0.05
- Delta value used to identify significant probesets: 1.24701
- Fudge factor for standard deviation computed: 0.04825.

Under these conditions, we identified a total of 3409 significant probesets corresponding to 2684 unique annotated genes (Fig. 1). We then selected probesets having ≥ 1.5 fold change difference (L1 vs. ctr) that resulted in a set of 496 upregulated and 743 downregulated probesets annotated, corresponding to 361 upregulated and 580 downregulated unique genes, referred to as the ‘L1-ECs signature’. Next, we uploaded the L1-ECs signature (probeset level) in the Ingenuity Pathway Analysis software (IPA, http://www.ingenuity.com) to identify biological functions/pathways putatively regulated by L1. The Mouse Gene 1.0 ST Array reference set present in the IPA database was used for the “IPA core analysis”. Only direct relationships in mammals (i.e., human, mouse, and rat) were considered, including endogenous chemicals for gene network analysis. The p-values for biofunction enrichment were corrected for multiple testing using the Benjamini-Hochberg correction. With these settings we identified 23 biofunctions significantly enriched (p-value < 0.05; Benjiamini-Hochberg correction) in L1-overexpressing cells compared to mock-transfected cells [1]. Considering the massive changes in transcriptional activity and the predicted effect on a variety of biological functions upon L1 overexpression, we then asked whether L1 could activate/inhibit transcription factor(s) (TFs). To address this question, we performed the “upstream regulator analysis” in the IPA. Such an analysis allows the identification of transcriptional regulators activated/inhibited under specific experimental conditions, thus accounting for the observed gene expression changes. Strikingly, we identified a total of 18 and 11 TFs predicted to be activated or inhibited by L1, respectively. Of note, 5 TFs were differentially regulated in L1-overexpressing vs. mock-transfected cells and, in particular, four of the activated TFs were upregulated in L1 overexpressing cells (STAT1, STAT2, IRF7 and ATF4), while one of the inhibited TFs was downregulated (FOXM1).

We then performed the Mechanistic Networks analysis to further explore the contribution of the TFs in regulating gene networks. We found that STAT1, STAT2, IRF7, ATF4 and STAT3 (that was among the 18 TFs predicted to be activated) interact with each other (Fig. 2), thus exerting a coordinated control on a directional network of 105 genes regulated by L1 (i.e. 11% of all the L1-regulated genes). Most of these genes were consistently up- or downregulated with the expression change of their upstream TFs [1]. These findings were supported by functional studies which implicated the JAK/STAT signaling pathway in the biological response of luECs to L1 overexpression [1].

**Fig. 1.** Significant Analysis of Microarrays (SAM) of luECs expression profile. In the y-axes, \( d(i) \) is the observed strength of the relationship between gene expression and the response variable (i.e. L1 overexpression). In the x-axes, \( d_E(i) \) is the expected strength (calculated by random permutations of data) of the relationship between gene expression and the response variable. Red dash lines represent the threshold \( d(i) - d_E(i) \) used to call significant genes. The \( \Delta \) threshold was adjusted to allow proportion of falsely called genes set by q-value of 0.05. In red, genes downregulated in L1-overexpressing luEC vs. mock-treated cells. In green, genes upregulated in L1-overexpressing IEC vs. mock-treated cells.

**Fig. 2.** Molecular interaction network of the upstream modulators found by IPA. Red labels indicate gene expression changes upon L1 overexpression (fold change). Number in brackets indicates the number of published reports supporting the molecular interaction. The type of interaction is also indicated (PP, protein–protein; E, expression; TR, translocation; RE, reaction; T, transcription; A, activation; I, inhibition).
Discussion

Here we describe the methods of analysis that were used to define and characterize the gene expression profile induced by L1 in murine lung endothelial cells (LuECs). We used the Affymetrix Mouse Gene 1.0 ST Arrays, which enabled us to produce gene expression data of a total of 28,869 genes (764,885 distinct probes). Through the combined use of the Significant Analysis of Microarrays (SAM) and the Ingenuity Pathway Analysis (IPA), we identified a set of 941 genes and 23 Bio-functions significantly regulated in L1-overexpressing vs. control LuECs. We reasoned that these massive transcriptional changes could be the result of the L1-mediated regulation of TFs. Indeed, by using the “upstream modulator analysis” in IPA we found that 29 TFs were predicted to be activated/inhibited and, among these, 5 TFs were transcriptionally regulated in our profiling experiment. Among the 29 TFs identified, many are involved in the inflammatory and/or angiogenic cascades (STAT1, STAT2, STAT3, IRF1, IRF3, IRF7, RELA/p65, NFKB1 and ATF4). Of note, while L1 has been causally linked to NF-κB signaling in cancer cells [3], we are the first to report a crosstalk between L1 and STATs, IRFs and ATF4. Our data, therefore, point to L1 as a novel orchestrator of the cancer-associated response that entails the regulation of gene networks and biochemical cascades which, in turn, impact on the pathophysiology of cancer cells as well as tumor microenvironment.

Importantly, the results of our in silico analysis have been successfully validated experimentally, thus lending further support to their biological relevance.

Conflict of interest

Authors declare no conflict of interest.

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