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HDL activates expression of genes stimulating cholesterol efflux in human monocyte-derived macrophages

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Abstract

High density lipoproteins (HDL) are key components of reverse cholesterol transport pathway. HDL removes excessive cholesterol from peripheral cells, including macrophages, providing protection from cholesterol accumulation and conversion into foam cells, which is a key event in pathogenesis of atherosclerosis. The mechanism of cellular cholesterol efflux stimulation by HDL involves interaction with the ABCA1 lipid transporter and ensuing transfer of cholesterol to HDL particles. In this study, we looked for additional proteins contributing to HDL-dependent cholesterol efflux. Using RNAseq, we analyzed mRNAs induced by HDL in human monocyte-derived macrophages and identified three genes, fatty acid desaturase 1 (*FADS1*), insulin induced gene 1 (*INSIG1*), and the low-density lipoprotein receptor (*LDLR*), expression of which was significantly upregulated by HDL. We individually knocked down these genes in THP-1 cells using gene silencing by siRNA, and measured cellular cholesterol efflux to HDL. Knock down of FADS1 did not significantly change cholesterol efflux ($p=0.70$), but knockdown of INSIG1 and LDLR resulted in highly significant reduction of the efflux to HDL (67% and 75% of control, respectively, $p<0.001$). Importantly, the suppression of cholesterol efflux was independent of known effects of these genes on cellular cholesterol content, as cells were loaded with cholesterol using acetylated LDL. These results indicate that HDL particles stimulate expression of genes that enhance cellular cholesterol transfer to HDL.

Keywords

HDL; monocyte-derived macrophages; cholesterol efflux; transcriptome analysis; lipid metabolism; atherosclerosis

Introduction

Cellular content of free (unesterified) cholesterol is tightly controlled and kept within a narrow range [1]. Changes in cholesterol content severely affect fluidity of cell membranes and impair various metabolic pathways by influencing kinetic of reactions catalyzed by membrane-associated enzymes. Increase or decrease of cellular cholesterol content beyond a set limit results in apoptosis or necrosis [1]. Extrahepatic cells show very limited catabolism of cholesterol, so that cellular cholesterol content is determined by cholesterol delivered (including *de novo* synthesis) and released from cells.

Several major pathways of cellular cholesterol efflux have been described. The ABCA1-dependent pathway involves cholesterol transfer to small HDL particles, including preβ1-HDL (also known as “lipid-poor” apoA-I) [2, 3]. The actual mechanism of this process is still subject to debate. The initial model for ABCA1mediated lipid efflux proposed by Fielding et al. included a two-step mechanism in which ABCA1 mediates phospholipid efflux to ApoA-I followed by efflux of free cholesterol [4]. Translocation of phospholipids via ABCA1 may create conditions on the membrane for apoA-I binding and cholesterol removal to HDL [5]. However, this model was disputed by Smith et al. who proposed that ABCA1 mediates a concurrent free cholesterol and phospholipid efflux to ApoA-I [6]. In the absence of ABCA1, cells are unable to transfer lipids to lipid-free/lipid-poor apoA-I and release excessive cholesterol. Selective disruption of ABCA1 in macrophages leads to

impairment of cholesterol efflux [7], accumulation of cholesterol in these cells and development of atherosclerosis [8].

Cholesterol transfer to HDL is tightly associated with maturation of HDL particles [9]. Hepatic and intestinal ABCA1-mediated cholesterol efflux to ApoA-I is a key event in HDL biogenesis. The major cholesterol efflux pathway in macrophages is ABCA1-mediated cholesterol efflux to small HDL particles [3]. Another cholesterol transporter, ABCG1, mediates cholesterol transfer to the lipidated mature HDL but not to lipid-free/lipid-poor apoA-I [10]. ABCA1 and ABCG1 work in tandem, with ABCA1 lipidating lipidpoor apoA-I and ABCG1 continuing lipidation by adding extra cholesterol [11]. However, it has been reported that ABCG1 does not promote cholesterol efflux from human macrophages [12]. Other ABC transporters, such as ABCA7 or ABCA12, have been suggested to contribute to cellular cholesterol efflux [13, 14], although Abca7 null mice retain normal macrophage phosphatidylcholine and cholesterol efflux activity[15]. Yet another efflux pathway involves scavenger receptor B1 (SCARB1 or SR-B1), which has been shown to participate in cholesterol efflux from human macrophages [12, 16]. SR-B1 was originally described as an HDL receptor responsible for selective removal of cholesteryl esters from HDL in the liver and steroidogenic tissues, but its contribution to cholesterol efflux from peripheral cells has also been suggested [17]. Transfection of cells with SR-B1 stimulates cholesterol efflux to mature HDL [18], but also induces cholesterol uptake [19]. It was suggested that SR-B1 is responsible for “docking” of HDL to the cell surface, which may result in either selective uptake of cholesteryl esters or cholesterol efflux by other energy-independent pathways depending on cell type and possibly metabolic state [20].

A number of genetic and biochemical studies demonstrated that cholesterol transfer from ABCA1 to HDL’s apoA-I is the main pathway of cholesterol efflux in many cell types, including macrophages [21]. Although direct interaction between ABCA1 and apoA-I has been suggested based on chemical cross-linking experiments [22, 23], the molecular mechanism of ABCA1-mediated lipid export and associated nascent HDL formation remains controversial. A recent paper defined the cryo-EM structure of human ABCA1 at 4.1Å resolution, which suggested a “lateral access” mechanism for ABCA1-mediated lipid export, where lipids are transferred from ABCA1 to apoA-I laterally in the extracellular space [24], rather than by a conventional alternating access paradigm (reviewed in [25]). Neither model, however, provides a complete list of proteins participating in HDL biogenesis, leaving open a possibility that additional factors may be involved in regulation of cholesterol efflux to HDL. In this study, we found that macrophages exposed to HDL upregulate expression of two proteins, INSIG1 and LDLR, that in turn regulate cholesterol efflux.

Results and Discussion

Our original objective was to identify genes in monocyte-derived macrophages (MDM) that are upregulated by HDL treatment and could participate in macrophage cholesterol efflux mechanisms. Two RNA samples from MDM obtained from a healthy donor were analyzed. One sample was from MDM differentiated in the presence of M-CSF, and the other – from MDM differentiated in the presence of GM-CSF. Whereas both growth factors promote

differentiation of monocytes into macrophages, based on expression of certain cytokines, the population resulting from GM-CSF treatment of human monocytes has been referred to as macrophages with a “pro-inflammatory” cytokine profile and with some features of M1 cells, whereas the M-CSF–generated counterparts displayed an “anti-inflammatory” cytokine repertoire and presented features of M2 macrophages [26]. Of note, macrophages obtained after M-CSF and GM-CSF differentiation in the absence of added cytokines do not represent fully polarized M2 and M1 cells. Since both growth factors are present under physiological conditions, we were interested in genes common to both differentiation treatments. To identify genes specifically upregulated by HDL, we used general linear model (glm) approach implemented in the edgeR package [27]. This package employs linear regression to remove differences induced by variability between phenotypes, allowing to take into account multiple types of batches, and assuming negative binomial model for regression error. We contrasted gene expression in HDL-activated macrophages against non-activated cells and macrophages from three other donors activated by native, acetylated or oxygenated low-density lipoproteins (total 16 samples). This analysis produced 67 differentially expressed genes (Supplementary Table 1). Only three genes (fatty acid desaturase 1, *FADS1*; insulin induced gene 1, *INSIG1;* and the low-density lipoprotein receptor. *LDLR*) displayed expression increasing for more than two-fold (logFC >1) in HDL-stimulated samples at the sufficiently high expression level (logCPM >5) and false discovery rate (FDR) less than 1 (highlighted in Supplementary Table 1).

The mechanisms through which HDL modulate gene expression in human macrophages were not investigated in the present study. The increased *LDLR* expression likely resulted from the reduction of the cellular cholesterol content in macrophages due to HDL-mediated cholesterol efflux. Reduced cholesterol content stimulates *LDLR* expression through SREBP2-SCAP pathway [28]. Similar mechanisms may occur for *INSIG1* and *FADS1*, which are also regulated through SREBP2 pathway [29, 30]. Since total HDL, containing the whole spectrum of HDL particles, was used in our experiments, both the net depletion of cellular cholesterol (through cholesterol efflux) [31] as well as the activation of cellular signaling pathways through HDL-associated phospholipids and lysosphingolipids (such as S1P [32]) may have contributed to the observed effects. Given that HDL particles vary between the donors in HDL size and composition, it is likely that the influence on cellular gene expression response may also vary between the donors. This could explain differences in atheroprotective function of HDL from different subjects. Future studies will be needed to investigate this interesting possibility.

We then proceeded to experimentally test whether identified genes have an effect on cellular cholesterol efflux. Indeed, if these genes are upregulated in response to HDL-mediated depletion of intracellular cholesterol with a purpose to replete the cholesterol content, it is reasonable to expect that they may also suppress the cholesterol efflux. As a model here, we used human THP-1 cells that can be differentiated into macrophage-like cells by PMA treatment. We knocked down individual genes of interest in THP-1 macrophages by siRNA (Fig. 1A), and analyzed cellular cholesterol efflux to HDL. HDL, rather than apoAI, was used as an acceptor because we aimed to determine the cumulative effect of identified genes on cellular cholesterol efflux, including efflux to small HDL particles. This assay measures a combined effect of cholesterol efflux and non-specific exchanges between the HDL

cholesterol and radiolabeled cellular cholesterol, which do not reduce cellular cholesterol content. However, such non-specific exchange is likely to be similar in all knockdown experiments, as cells were pre-loaded with cholesterol. Results presented in Fig. 1B demonstrate that knockdown of *FADS1* did not affect cholesterol efflux. In contrast, knockdown of *INSIG1* or *LDLR* downregulated efflux of cholesterol to HDL acceptor (Figs. 1C and 1D). Experiments were performed 3–4 times with each condition. To account for variability in cholesterol efflux between the replicates in different experiments, cumulative results are presented as cholesterol efflux normalized to the efflux from control cells transfected with scrambled siRNA (Fig. 1E). Statistical analysis performed on all data demonstrated significant differences between the efflux from control cells and cells with downregulated *INSIG1* and *LDLR*, but not *FADS1*.

The observed effect of *INSIG1* knockdown on cellular cholesterol efflux was counterintuitive, given that INSIG1, together with INSIG2, mediates degradation of HMGR protein and the retention of SREBP in the ER, providing a control mechanism that prevents the accumulation of sterols within the cell [33–35]. Thus, *INSIG1* knockdown is expected to result in lipid accumulation, as demonstrated *in vitro* and *in vivo* [36], and compensatory stimulation of cholesterol efflux. Inhibition of cholesterol efflux observed here would exacerbate lipid accumulation.

LDLR regulates the intracellular cholesterol levels by mediating the intake of plasma-derived LDL cholesterol, so reduced *LDLR* expression is expected to result in reduced intracellular cholesterol and diminished cholesterol efflux [37]. However, THP-1 macrophages in our experiment were loaded with cholesterol using acLDL, which does not bind to LDLR. Therefore, *LDLR* knockdown cannot significantly affect the cellular cholesterol content in these experimental conditions.

In view of discussed above arguments, we hypothesized that reduction of cholesterol efflux in cells with knocked down *INSIG1* and *LDLR* genes was due to associated suppression of expression of cholesterol transporters, namely *ABCA1*, *ABCG1*, and *SCARB1*. We analyzed expression of these three genes using the same RNA that was used for testing knock-down of *INSIG1*, *LDLR*, and *FADS1*. This analysis demonstrated that *ABCA1* (Fig. 1F) and *ABCG1* (Fig. 1G) were downregulated in cells with knocked-down *INSIG1* and *LDLR*, but not in cells with knocked-down *FADS1*. In contrast, *SCARB1* was not downregulated in any condition (Fig. 1H). Unfortunately, only two replicates were available for this analysis, preventing statistical confirmation. Nevertheless, these results suggest that downregulation of *ABCA1* and *ABCG1* is responsible for reduced cholesterol efflux in cells with knocked-down *INSIG1* and *LDLR* genes.

The mechanism of this interesting effect remains to be investigated. Off-target effects of siRNAs used for gene knock-down are unlikely, given that these siRNAs were purchased from a commercial vendor (Invitrogen) and were subjected to strict quality control, and no homology is found between *INSIG1* or *LDLR* and *ABCA1* or *ABCG1* genes in the regions targeted by siRNAs. The known links between expression of *INSIG1* and *LDLR* and that of *ABCA1* and *ABCG1* revolve around changes in cellular lipid content [25, 38–40]. In particular, downregulation of *INSIG1* and *LDLR* may alter levels of oxysterol or cholesterol

precursor, leading to reduced activation of LXR, which is the main regulator of *ABCA1* and *ABCG1* expression [41]. This mechanism is consistent with selective suppression of *ABCA1* and *ABCG1*, but not *SCARB1*, which is not regulated by LXR [41].

Previous studies analyzing transcriptome changes in macrophages treated with HDL focused primarily on inflammatory genes [42–44]. Here, we demonstrate that HDL stimulates expression of genes regulating cholesterol homeostasis, thus affecting cellular cholesterol efflux and reverse cholesterol transport. The positive effect on cholesterol efflux of HDL-stimulated genes identified in this study, INSIG and LDLR, is novel and counterintuitive, as it appears to oppose the classical reason for upregulation of these genes, which is replenishment of cellular cholesterol lost due to HDL-stimulated cholesterol efflux. A question for future studies concerns the mechanism of this HDL effect. HDL is known to induce a number of intracellular signalling pathways [45]. Whereas many of these pathways are activated by HDL-mediated cholesterol efflux and changes in intracellular cholesterol content, some others result from HDL interaction with plasma membrane proteins and cannot be reproduced by treatment with cholesterol acceptor methyl-βcyclodextrin [46]. Extension of the findings reported in the current study will advance our understanding of the anti-atherogenic activity of HDL and help identify new therapeutic agents fighting atherosclerosis.

Methods

Cell cultures

Monocytes were isolated from peripheral blood mononuclear cells by adherence to plastic, as described previously [47]. Monocytes were differentiated into macrophages (MDM) by incubating cells for 6 days in RPMI supplemented with 10% heat-inactivated human serum, 2 mM L-glutamine and 100 μg/ml penicillin/streptomycin and M-CSF or GM-CSF (25 ng/ml each). Human THP-1 monocytic cell line was obtained from American Type Culture collection (ATCC) and maintained in RPMI with 10% heatinactivated fetal bovine serum, 2 mM L-glutamine and 100 μg/ml penicillin/streptomycin. THP-1 monocytes were differentiated into macrophage-like cells by incubation for 3 days in medium supplemented with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL).

HDL and LDL preparation

Total HDL (density = 1.063–1.21 g/mL) and LDL (density 1.019–1.063 g/mL) were isolated from normolipidemic plasma of a healthy donor by preparative ultracentrifugation as previously described [48]. LDL acetylation reaction was performed under stirring on ice. Briefly, native LDL were acetylated with an equal volume of saturated Sodium Acetate (NaOAc) solution followed by the addition of acetic anhydride (0.75% of the initial volume) in small aliquots over a period of 1 hour. An additonal 30-minutes incubation enables the completion of the reaction. For tritium labelling, acetylated LDL (acLDL) sample was incubated with [^{3}H]cholesterol for 30 minutes at 37°C. Oxidized LDL was produced by incubating LDL (100 μg/ml) for 3 h in the presence of Cu^{2+} (5 μM) at room temperature, as previously described [49].

RNAseq and gene identification

MDM were incubated for 24 h with 15 μg/ml HDL, and total RNA was isolated. RNA-seq libraries were prepared using a NEBNext Ultra RNA library prep kit for Illumina according to the manufacturer's instructions. Libraries were PCR-amplified for 12–15 cycles, and sequenced on a HiSeq 1500 (Illumina).

To identify genes showing differential expression we used edgeR package [27]. For the initial preprocessing we dropped lowly expressed genes, keeping only genes with cpm values greater than 10 in at least three samples, arriving at total 9565 genes expressed in macrophages. To make the result more robust, we estimated the regression parameters with the inflated control dataset, using 16 samples in total, adding control macrophage samples from two other donors, as well as 10 samples of macrophages activated by native, acetylated and oxygenated low-density lipoproteins from three donors. We contrasted gene expression in HDL-activated macrophages against all other activation stimuli, considering the macrophage phenotype (M-CSF or GM-CSF), the donor, and the activation status (control or activation with different modifications of LDL) as extra variables.

Radioisotopic cholesterol efflux assay

Cellular cholesterol efflux from THP-1 macrophages to 15 μg phospholipid/ml total HDL about 30 μg/ml total protein was assayed in triplicate as previously described [50]. Cells were cholesterol loaded for 24 h with 50 μg/mL [^{3}H]cholesterol-labeled acetylated LDL (1 μCi/mL) in serum-free RPMI 1640 supplemented with 50 mM glucose, 2 mM glutamine, 0.2% BSA (RGGB medium) and 100 μg/ml penicillin/streptomycin. The labeling medium was removed and THP-1 macrophages were then equilibrated in RGGB medium for additional 16–24 h. Although during this period a portion of labelled free cholesterol is esterified to cholesterol esters and stored in lipid droplets, a significant portion of cholesterol is incorporated in plasma membrane [51], which is the primary site from which cholesterol efflux operates. Cellular cholesterol efflux was assayed in serum-free medium after a 4-hour chase period. The 4 h period allows a rapid and sensitive measurement of cholesterol efflux associated with the net export of cholesterol mass from the cell. If longer periods of incubation are used, additional cholesterol movements must be taken into account, including cholesterol efflux to apoE [52], which is secreted at high amounts by macrophages [53], as well as the uptake of HDL by macrophages. Culture media were harvested, cleared of cellular debris by a brief centrifugation, and radioactivity was counted in a liquid scintillation counter (Wallac Trilux 1450 Microbeta). Cell radioactivity was determined by extraction in hexane-isopropanol (3:2), evaporation of the solvent and liquid scintillation counting. Cellular cholesterol efflux was calculated as a percentage of radioactivity released from cells, notably as radioactivity in the medium/(radioactivity in the medium + radioactivity remaining in the cells) × 100%. Acceptor-specific efflux is the difference between the efflux to the serum-free medium with added acceptor (apoA-I or HDL) and medium without acceptor. Four to ten experiments were performed for each knockdown, and results are presented as cholesterol efflux normalized to the efflux from cells transfected with scrambled siRNA (control). Statistical analysis was performed by Student's two-tailed *t* test, with $p<0.05$ taken as significant.

RNA interference-mediated gene silencing using small interference (si)RNA

Knock-down of selected genes was achieved by transfecting THP-1 macrophages using lipofectamine RNAiMax (Invitrogen) according to manufacturer's instructions. Cells were transfected with 50 nM control scrambled siRNA (Invitrogen) or siRNA targeting genes of interest, *FADS1, INSIG1, and LDLR* (Santa Cruz Biotechnology).

Quantitative RT-PCR

To quantify siRNA-mediated knockdown, total RNA was extracted from siRNA-transfected THP-1 macrophages using a NucleoSpin RNA II kit (Macherey-Nagel) according to manufacturer's instructions. RNA was reverse transcribed using random hexamer and MMLV reverse transcriptase. An initial denaturation step for 5 min at 68°C was followed by an elongation phase of 1 h at 42°C; the reaction was completed by a 5-min incubation at 68°C. Real time quantitative PCR was performed in triplicate using a LightCycler LC480 (Roche). Each reaction contained 2.5 ng of reverse transcribed total RNA, 150 pmol of forward and reverse primers specific for the selected genes and 5 μl of Master Mix SYBR-Green, in a final volume of 10 μl. Crossing point (CP) values for genes of interest were normalized to housekeeping genes (human delta-aminolevulinate synthase and human alpha-tubulin). Expression data were based on the crossing points calculated with the software for LightCycler data analysis and corrected for PCR efficiencies of the target and the reference genes. Data were expressed as a fold change in mRNA expression relative to control values.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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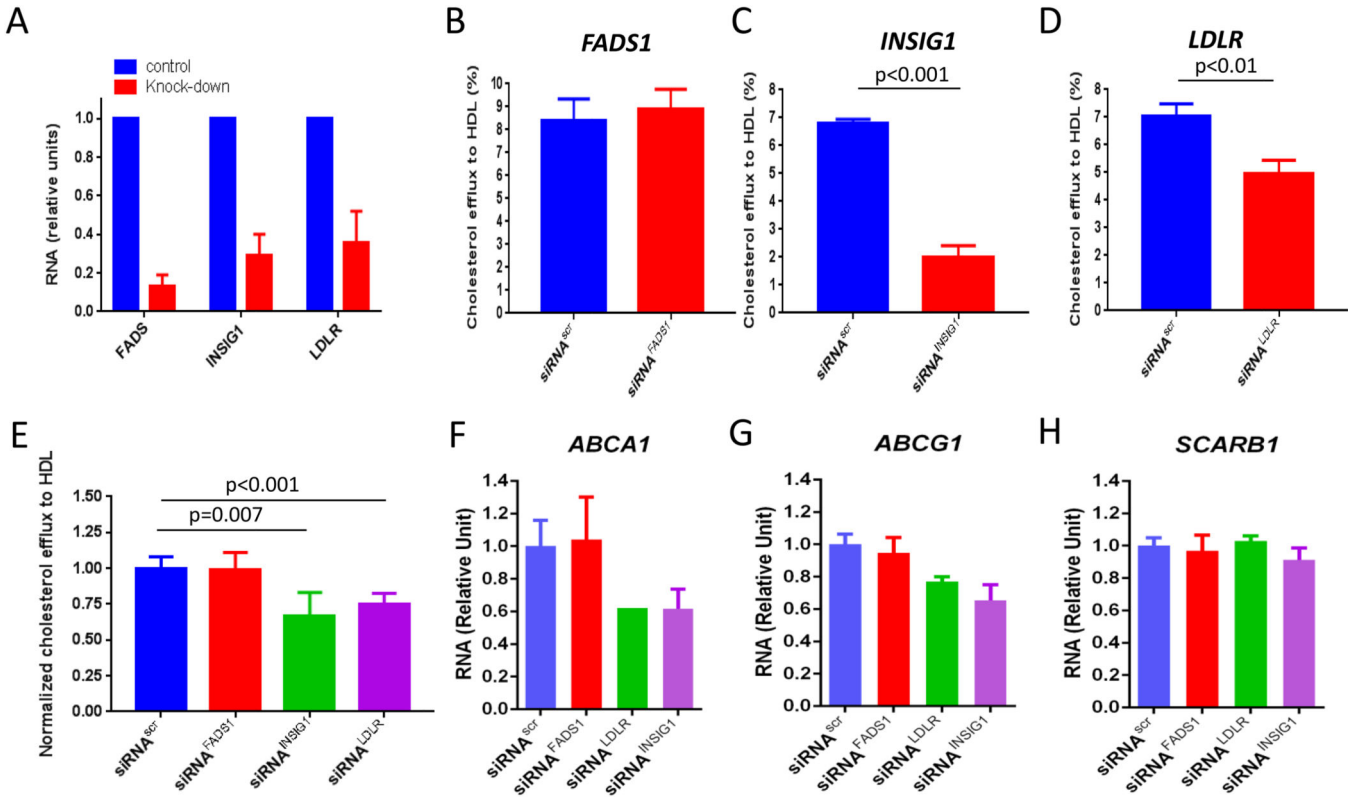

Figure 1. Analysis of cholesterol efflux from THP-1 cells with knocked-down genes.

A – Indicated genes were knocked down by transfecting PMA-differentiated THP-1 cells with siRNA. Control cells were transfected with scrambled siRNA. Results show mean ± SD of triplicate determinations. B – Representative experiment analyzing cholesterol efflux to HDL from cells transfected with scrambled or *FADS1*-targeting siRNA. Results show mean ± SD of triplicate determinations. C – Representative experiment showing cholesterol efflux to HDL from cells transfected with scrambled or *INSIG1*-targeting siRNA. Results show mean ± SD of triplicate determinations. D - Representative experiment showing cholesterol efflux to HDL from cells transfected with scrambled or *LDLR*-targeting siRNA. Results show mean ± SD of triplicate determinations. E – Cumulative results of cholesterol efflux experiments showing cholesterol efflux normalized to efflux from control cells. N=14 for control cells, n=3 for *FADS1* KD cells, n=4 for *INSIG1* KD cells, and n=4 for *LDLR* KD cells. F, G, H – Analysis of *ABCA1, ABCG1, and SCARB1* RNA in cells with knocked-down genes used in panel A. Results show mean ± SEM of duplicate determinations