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bjh british journal of haematology

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| Journal: | *British Journal of Haematology* |
| Manuscript ID: | BJH-2009-01865.R1 |
| Manuscript Type: | Ordinary Papers |
| Date Submitted by the Author: | 17-Jun-2010 |
| Complete List of Authors: | Choudhury, Aniruddha; Department of Oncology-Pathology, Karolinska Institutet<br>Derkow, Katja; Department of Oncology-Pathology, Karolinska Institutet<br>Danesh-Manesh, Amir Hossein; Department of Oncology-Pathology, Karolinska Institutet<br>Mikaelsson, Eva; Department of Oncology-Pathology, Karolinska Institutet<br>Kiaii, Shahryar; Department of Oncology-Pathology, Karolinska Institutet<br>Kokhaei, Parviz; Department of Oncology-Pathology, Karolinska Institutet<br>Osterborg, Anders; Department of Oncology-Pathology, Karolinska Institutet; Departments of Hematology and Oncology, Karolinska University Hospital Solna<br>Mellstedt, Hakan; Department of Oncology-Pathology, Karolinska Institutet; Departments of Hematology and Oncology, Karolinska University Hospital Solna |
| Key Words: | APOPTOSIS, CHRONIC LYMPHOCYTIC LEUKAEMIA, TUMOUR ANTIGENS |
| | |
| Note: The following files were submitted by the author for peer review, but marked to be sent in Off-Line. | |
| Suppl Fig 1A_1B.tif | |

# Silencing of Ror-1 and Fibromodulin with siRNA results in apoptosis of CLL cells

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Running title: siRNA-mediated silencing of Ror-1 and fibromodulin

Authorships

AC, AÖ and HM designed research. AC, KD, AHD, EM, SK and PK performed research. All co-authors contributed vital new reagents or analytical tools. AÖ collected samples and all co-authors analyzed and interpreted data, performed statistical analysis, and wrote the manuscript.

**Abstract**

We have previously demonstrated that Ror1 and fibromodulin (FMOD) are two genes upregulated in CLL cells compared to normal blood B cells. In this study, we have used siRNAs to specifically silence Ror1 and fibromodulin gene expression in CLL cells, healthy B cells and human fibroblast cell lines. siRNA treatment induced a specific reduction (75–95%) in fibromodulin and Ror1 mRNA. Western blot analysis with specific antibodies for fibromodulin and Ror1 demonstrated that the proteins were significantly downregulated 48 hours after siRNA treatment. Silencing of fibromodulin and Ror1 resulted in statistically significant ($p \leq 0.05$-$0.001$) apoptosis of CLL cells but not of B cells from normal donors. Human fibroblast cell lines treated with fibromodulin and Ror1 siRNA did not undergo apoptosis. This is the first report demonstrating that Ror1 and fibromodulin may be involved in the survival of CLL cells. Ror-1 in particular is further explored as potential target for therapy in CLL.

Keywords: Ror1, fibromodulin, siRNA, apoptosis, CLL, silencing

## Introduction

Targeted anticancer therapies depend on the discovery of molecules that are uniquely expressed or overexpressed by malignant cells and are critical for the growth and survival of the cancerous cells. Gene array analyses have previously demonstrated that the genes Ror1 and fibromodulin (FMOD) are upregulated in chronic lymphocytic leukemia (CLL) cells (Jelinek, *et al* 2003, Klein, *et al* 2001, Vallat, *et al* 2003). Several studies, including our own have subsequently confirmed that Ror1 and fibromodulin are expressed by CLL cells but not by normal nonmalignant B cells (Baskar, *et al* 2008, Daneshmanesh, *et al* 2008, Fukuda, *et al* 2008, Mayr, *et al* 2005, Mikaelsson, *et al* 2005).

Fibromodulin is a 59 kDa collagen-binding protein of the leucine-rich repeat family of proteins located on chromosome 1q32.1. It is found in many types of connective tissues such as cartilage, sclera, tendon, skin and cornea (Oldberg, *et al* 1989). This protein is thought to be involved with extracellular matrix organization by means of its ability to interact with collagen (Hedbom and Heinegard 1989). fibromodulin is also known to bind TGF-β and modulate its biological activity (Hildebrand, *et al* 1994, Soo, *et al* 2000).

Ror1 is located on the chromosomal region 1p31.3 (http://www.ensembl.org) and is a 105 kDa protein with potential tyrosine kinase function (Yoda, *et al* 2003). Ror1 mRNA is normally expressed in heart, lung and kidneys in adult humans and to a lesser extent in the placenta, pancreas and skeletal muscles and a truncated Ror1 is known to be expressed in human leukemia and lymphoma cell lines (Reddy, *et al* 1996). Recent studies have demonstrated that Ror1 can bind Wnt5a in CLL cells (Fukuda, *et al* 2008) and is currently being actively explored *in vitro* by several research groups as a potential target for therapy of CLL.

Using a large cohort of patients we have previously demonstrated that Ror-1 and fibromodulin are overexpressed at the protein level in virtually all patients with CLL tested and is not detected in circulating B cells and other peripheral blood cells in normal donors (Daneshmanesh, *et al* 2008, Mikaelsson, *et al* 2005). In the present study we have used siRNAs to specifically silence these two genes in CLL cells and examine their role in survival of CLL cells.

## Materials and Methods

Blood samples: Blood was obtained from totally thirteen CLL patients and two healthy donors with informed consent. Peripheral blood was drawn in heparinized tubes according to protocols approved by the regional ethics committee and in accordance with the Declaration of Helsinki.

None of the patients were receiving anti-leukemic therapy when their blood was collected. Three patients had progressive CLL and had previously received fludarabine-based chemotherapy (9-26 mo before sampling). Their leukemic cell count ranged from 90–236 x $10^9$/L. Ten patients had indolent CLL, (Rai stage 0-1) with leukemic cells between 17 and 53 x $10^9$/L.

Mononuclear cells were separated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density-gradient centrifugation. B cells from the blood of healthy donors were further enriched by filtration through nylon wool. (Rezvany, *et al* 2000)

Cell lines: The adherent human fibroblast cell lines HFSF-PI3, HSF-PI19, HFFF PI6, and HSF-PI18 were gifts from the National Cell Bank of Iran. These, as well as NIH-3T3 fibroblasts stably transfected to express membrane-bound human CD40L (Microbiology and Tumor Biology Centrum, Karolinska Institute, Sweden) was cultured in RPMI 1640 (HFFF PI6, and HSF-PI18, 3T3) or in a 1:1 mixture of DMEM and IMDM (Gibco, Paisley, Scotland). Media were supplemented with 10 or 20% FBS (Gibco) 1% L-Glutamine (2mM) and 1% Pen-Strep (Gibco). Adherent cells were removed with trypsin-EDTA (Gibco), washed and resuspended in fresh media prior to use in experiments. Viability of cells was determined by quantifying the number of cells in a Buerker Chamber (hemocytometer), mixed in a dilution of 1:10 with 0.4% trypan blue (Gibco)

Oligonucleotides and primers

siRNA against fibromodulin and Ror1 were designed using a commercially available algorithm (www.dharmacon.com). Three separate siRNA were designed against distinct regions of each gene. Control non-silencing siRNA were also purchased from Dharmacon Inc. (Lafayette, CO, USA). All siRNA were supplied as dried pellet in presence of buffer by the vendor. The siRNA

were suspended, aliquoted and cryopreserved at -20$^{o}$C according to manufacturer's instructions. Primers for polymerase chain reaction (PCR) were obtained from CyberGene AB, Novum Research Park, Sweden. The sequence of siRNA and PCR primers are provided in Table 1A-B. CLL cells were transfected with three different siRNAs each against fibromodulin or Ror1. Additionally, the cells were also transfected with a pool of the fibromodulin or Ror1 siRNA containing the three at equal concentrations. Two controls were included in every experiment; untransfected cells and cells transfected with a pool of 4 different non-silencing control siRNA.

Transfection of cells with siRNA

The day before transfection, CD40L$^{+}$-3T3 fibroblasts were added to 24 well plates at a concentration of 250,000 cells/well and incubated at 37 $^{o}$C in 5% $CO_2$ and 98% humidity. For every experiment PBMC from normal donors or CLL patients were freshly ficolled, counted and suspended in Optimem® (Invitrogen, Carlsbad, CA) at a concentration of 10x10$^{6}$ viable cells/ml. 2 x 10$^{6}$ cells in 200 µls of Optimem® were added to each well of 48-well plates for siRNA transfection.

Cells were transfected using the cationic, nonliposomal transfection reagent TransIT-TKO® (Mirus Bio LLC, Madison, WI). Details of the quantities of reagents utilized are provided in table 2 and are adapted from the published protocol provided by the manufacturer of the transfection reagents. In brief, the diluted transfection reagent was mixed with an appropriate amount of siRNA, incubated for 10 minutes at RT. The siRNA/transfection reagent complex was then added dropwise to the cells in the well of the 48-well plate. The plate was incubated at 37$^{o}$C in 5% $CO_2$ and 98% humidity for 6 hours with gentle rocking every hour. At the end of 6 hours the cells were transferred to the washed CD40L$^{+}$-3T3 fibroblast monolayers and further incubated with an additional 1 ml of AIM V® till the termination of the experiment.

The human fibroblast cell lines were transfected with siRNA using a procedure that is essentially similar to the one described above with minor modifications. The fibroblast cell lines were added at the concentration of 50,000 cells/well in a 24-well plate and 240,000 cells/ well in a 6-well plate on the day prior to transfection with siRNA. Transfection was performed as described

above and the cells were subsequently cultured by themselves rather than with $CD40L^+$-3T3 fibroblast.

Apoptosis quantification by flow cytometry

Apoptosis in cells was measured by flow cytometry following staining with Annexin-V and propidium iodide. Cells were stained with anti-CD3 antibody conjugated with APC (BD Biosciences, San Jose, CA) prior to staining with Annexin-V and propidium iodide. A commercially available kit was used for staining cells (BD). Cells were gently detached from the $CD40L^+$-3T3 fibroblast layer, collected in a tube, washed with PBS and 100µls of the cell suspension was stained with 5µls of Annexin V-FITC and 5µls of propidium iodide. Cells were examined using a FACScalibur (BD) flow cytometer and analyzed using the CellQuest™ software.

Polymerase chain reaction (PCR)

RT-PCR and quantitative RT-PCR was performed as described previously (Daneshmanesh*, et al* 2008, Mikaelsson*, et al* 2005) with minor modifications. RNA was extracted using a commercial kit (RNeasy, Qiagen Inc., Valencia, CA, USA). Cells were homogenized by repeated passage through a 22G needle. DNA contamination was minimized by DNase1 treatment (Qiagen). RNA samples were stored at -80$^{o}$C. Following reverse transcription as described previously (Mikaelsson*, et al* 2005), the cDNA was amplified for 30 cycles for the housekeeping genes β-actin and RPLP0 and 35 cycles for fibromodulin and Ror1.

Cell lysis and Western Blotting

Cells were lysed directly in the 24-well plate adding 60 µL of lysis buffer containing 0.2% triton-X, 130 mM HEPES, 4 mM MgCl2, 10 mM EGTA with 2% proteinase inhibitor cocktail (Sigma, St Louis, MO, USA)fibromodulin to each well. After 1 h incubation on ice, lysates were harvested and centrifuged at 2500 rpm for 5 min and the soluble fraction was collected. 30 µL of each sample was loaded onto a 10% NuPAGE Bis-Tris gel (Invitrogen, Carlsbad, CA, USA) and run at 120 V for 3 h under reducing conditions. Resolved proteins were transferred onto Immobilon-P PVDF membrane (Millipore Corporation, Bedford, MA, USA) in a Mini-Transblot Cell (Invitrogen). The membranes were blocked at room temperature for one hour with 5% non-fat

milk (Semper, Stockholm, Sweden) in PBS plus 0.05% Tween 20 (PBS-T). Filters were incubated with 2 μg/ml of anti-FMOD mouse monoclonal antibody (R&D, Minneapolis, MN, USA) over night at +4$^{o}$C. Following extensive washings in PBS-T, filters were incubated with a secondary horseradish peroxidise (HRP)-conjugated rabbit anti-mouse antibody (DakoCytomation, Glostrup, Denmark) for 1.5 h at room temperature. Filters were developed using Amersham Enhanced Chemiluminescence ECLTM system (GE Healthcare, Bio-sciences AB, Buckinghamshire, UK). To verify equal loading of samples, filters were stripped in a buffer containing 62.5 mM Tris-HCL, 2% SDS, 100 mM Mercaptoethanol (Sigma) at 50$^{o}$C for 30 min. Following 3x15 min washing in PBS-T, the membranes were re-probed with 2.5 μg/ml of a mouse anti-β-actin monoclonal antibody (Sigma).

Western blot for Ror1 was performed essentially as described above with minor modifications (Daneshmanesh et al, 2008). Ror1 affinity purified antibody (R&D Systems) was used at a concentration of 1μg/ml as the primary antibody.

**Results**

CLL cells can be transfected with siRNA at a high efficiency

In initial experiments we validated the efficiency of our siRNA transfection method. The siGLO® (Dharmacon) is a stable, fluorescent, non-targeting control siRNA used to establish optimum conditions for transfection for maximum siRNA uptake. CLL cells from 6 different patients were transfected with siGLo® siRNA and examined by flow cytometry at various time points after transfection for percentage of fluorescent cells. The results are provided as supplementary information. Using the transfection procedure described in the Materials and Methods section, >90% of CLL cells had initial uptake of siGLO® siRNA and the percentage of fluorescent cells diminished slowly over a period of 72 hours.

Silencing of Ror1 and fibromodulin and their effects on CLL cells

CLL cells *in vivo* are characterized by accumulation of mature lymphocytes that are resistant to apoptosis, but they tend to rapidly undergo spontaneous apoptosis *in vitro* (Collins*, et al* 1989). CD40 expression has been found on 90% to 100% of CLL cells and *in vitro*, activation of CD40 by

its natural ligand, CD40L, or by activating monoclonal antibodies, has been shown to support the survival and expansion of B-CLL cells (Grdisa 2003, Pedersen*, et al* 2002). We transferred the CLL cells on to a monolayer of CD40L-transfected fibroblasts, 6 hours after transfection, to minimize spontaneous apoptosis that tended to obscure the effects of Ror1 and fibromodulin silencing. We used the same primers as for the human cells for RT-PCR analysis of the NIH-3T3 murine fibroblast cell lines, transfected with human CD40L. With these primers, no PCR product corresponding to Ror1 and fibromodulin was identified, ensuring that the expression of these genes was detected from the CLL cells rather than the feeder cells (data not shown). The feeder layer therefore facilitated investigating the effect of Ror1 and fibromodulin silencing without spontaneous apoptosis *in vitro*.

Transfection of CLL cells with siRNA against fibromodulin or Ror-1 resulted in a significant decrease in the expression of Ror1 or fibromodulin gene as measured by RT-PCR. Figure 1 demonstrates the silencing of Ror1 and fibromodulin as detected by RT-PCR. A major downregulation of Ror1 and fibromodulin mRNA but not of the housekeeping (control) gene was observed at 24 hours following siRNA transfection. The siRNA-mediated downregulation was quantified by real-time PCR. Figure 2 represents CLL cells from three patients and in each case the gene expression of untransfected and siRNA-transfected cells are compared to that of by cells transfected with the control, non-silencing siRNA, which is considered to be 100%. The real-time PCR results demonstrated that the siRNA constructs silenced Ror1 and fibromodulin gene expression by 75->95% (Figure 2).

Downregulation of fibromodulin and Ror1 could also be demonstrated at the protein level by Western blotting. Figure 3a demonstrates downregulation of the fibromodulin bands in 3 CLL patients following silencing with FMOD-specific siRNA (lanes 3-5) compared to untransfected (lane 1) or control, non-silencing siRNA (lane 2) treated cells. Beta-actin levels, used as a control is below. Similar results were also noted for Ror1 following transfection of CLL cells from three patients with Ror1-specific siRNA (Figure 3b). The 105KDa band corresponding to Ror1 was detected in untransfected and control-transfected CLL cells (Lanes 1 and 2 respectively) but was absent in CLL cells treated with Ror1-specific siRNA. Levels of β-actin are

shown below the Ror1 panels. The three patient samples used in the protein down regulation experiments were also examined for apoptosis and the results are included subsequently (see Figure 5).

Silencing of Ror1 and fibromodulin genes resulted in profound changes in the biological function of CLL cells. Visualization by light microscopy showed that the CLL cells transfected with siRNA against Ror1 and fibromodulin had an observable change in morphology. Compared to untransfected cells or those transfected with control non-silencing siRNA, cells transfected with Ror1 or fibromodulin siRNA aggregated together in large dense clusters and appeared to be more granular than the control cells (Figure 4). Concomitantly, CLL cells treated with Ror1 and fibromodulin siRNA underwent apoptosis. Figure 5 demonstrates apoptosis induction in CLL cells of seven patients following transfection with siRNA against fibromodulin (A) and Ror-1 (B). The level of apoptosis induction following siRNA treatment varied between different patients as was the extent of spontaneous apoptosis but in all 7 patients level of apoptosis was markedly and significantly ($p \leq 0.05$-$0.001$; Student's paired, two-tailed t-test) higher with all 3 siRNAs than apoptosis with the control non-silencing siRNA (Figure 5). Time kinetics indicated that apoptosis was noted as early as 4 hours after transfection and was continuing at the end of 48 hours (data not shown).

To examine whether the effect of silencing Ror1 and fibromodulin was specific to CLL cells we tested the effect of siRNA transfection on PBMC of healthy donors and four human fibroblast cell lines. PBMC from two healthy donors were subjected to nylon wool filtration and both the B cell rich fraction recovered from the column and the T cell rich fraction in the effluent were transfected with siRNA. As seen in Figure 6, neither the siRNA pool against fibromodulin nor Ror1 induced apoptosis (<10%) in normal B and T cell enriched cell fractions. These results were in keeping with our earlier report that expression of fibromodulin and Ror1 is virtually absent on normal PBMC (Daneshmanesh*, et al* 2008, Mikaelsson*, et al* 2005). Of the four FMOD+ Ror1+ human fibroblast cell lines tested only HFFF-PI 6 demonstrated induction of apoptosis following transfection with pooled siRNA against fibromodulin and Ror1 (Figure 7). Interestingly, this is the only cell line of the four tested that is of fetal origin.

## Discussion

Our previous studies have demonstrated that Ror1 and fibromodulin are differentially expressed by CLL cells (Daneshmanesh*, et al* 2008, Mikaelsson*, et al* 2005). Studies by other investigators have revealed that fibromodulin may serve as a tumor –associated antigen that is naturally processed and presented in CLL. Mayr et al demonstrated that stimulation of autologous lymphocytes with native or CD40-ligand treated CLL cells resulted in the expansion of T cells capable of recognizing antigen peptides derived from fibromodulin (Mayr*, et al* 2005). RT-PCR examination by Giannopolous et al demonstrated that fibromodulin is one of the prominent TAAs expressed by CLL cells (Giannopoulos*, et al* 2006) while Hus et al demonstrated that vaccination with autologous dendritic cells loaded with CLL cell lysate increased the frequency of fibromodulin-reactive T cells (Hus*, et al* 2008). These reports indicate that fibromodulin may be relevant as a target for active immunotherapy which is further reinforced by the findings of our present study that fibromodulin appears to be important for the survival of CLL cells. However, the ubiquitous expression of fibromodulin in connective tissues necessitates caution due to the risk of autoimmune reactions. In spite of these reports however, the role of fibromodulin in the pathophysiology of CLL remains unclear. One of the putative mechanisms maybe through the sequestration of TGF-β(Lotz*, et al* 1994). Our preliminary experiments with the addition of neutralizing antibody to TGF-β or fibromodulin-positive fibroblasts could not confirm this hypothesis (data not shown). Alternatively, fibromodulin may facilitate the interaction of CLL cells with cells of the marrow stroma, peripheral blood derived “nurse-like” cells and extramedullary mesenchymal cells *in vivo* (Burger and Kipps 2002, Burger*, et al* 2000) that was exemplified by the interaction of the CLL cells with the CD40L-transfected 3T3 fibroblast monolayer in our experiments.

Like fibromodulin, little is presently known about the role of Ror1 in the pathophysiology of CLL. However, a recent study by Fukuda et al (Fukuda*, et al* 2008) provides evidence that Ror1 may serve as a receptor for Wnt5a signaling in CLL cells. In this report, the authors demonstrated that the extracellular domain of Ror1 could bind Wnt5a and coexpression of Ror1 and Wnt5a in HEK cells lead to the activation of NF-κB. Additionally the viability of CLL cells cocultured with Wnt5a-expressing CHO cells was significantly greater than those cultured with CHO cells alone.

This survival advantage was neutralized by the addition of antisera against Ror1. In yet another study, high-throughput RNAi screening was utilized to identify kinases and phosphatases in the human genome that are essential to the survival of cancer cells (MacKeigan*, et al* 2005). In the screen, 650 known and putative kinases were targeted by RNAi in HeLa cervival carcinoma cells. Of the 73 genes that were overall identified as survival kinases, Ror1 was one of four most potent survival kinases. Greater than three-fold increase in apoptosis was noted in HeLa cells, 72 hours after silencing of Ror1. These results taken cumulatively with those of the present study suggest that Ror1 may be an important survival kinase and targeting Ror1 using small molecules, therapeutic monoclonal antibodies or active immunotherapy approaches may be of utility for the treatment of CLL.

**Conflict of interest**

The authors declare that there is no conflict of interest.

**Acknowledgements**

This work was supported by the EUCAAD 200755 funded by the European Community's Seventh Framework Programme, The Swedish Cancer Society, CLL Global Research Foundation, Cancer Society of Stockholm, Cancer and Allergy Foundation and the King Gustaf V Jubilee Fund.

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## Tables

Table 1A: Target and siRNA sequences for fibromodulin and Ror1

| Target Sequence | siRNA Sequence | siRNA name |
|---|---|---|
| Fibromodulin: A TAG GAC CTA CGA GCC TTA | Sequence:<br>A.U.G.A.G.A.C.C.U.A.C.G.A.G.C.C.U.U.A.dT.dT<br>Complement:<br>U.A.A.G.G.C.U.C.G.U.A.G.G.U.C.U.C.A.U.dT.dT | FMOD 1 |
| Fibromodulin: A CAA TCG CAA CCT CAA GTA | Sequence:<br>A.C.A.A.U.C.G.C.A.A.C.C.U.C.A.A.G.U.A.dT.dT<br>Complement:<br>U.A.C.U.U.G.A.G.G.U.U.G.C.G.A.U.U.G.U.dT.dT | FMOD 2 |
| Fibromodulin: T GTA CCT CCA ACA GCA TGA | Sequence:<br>U.G.U.A.C.C.U.C.C.A.A.C.A.C.G.A.U.G.A.dT.dT<br>Complement:<br>U.C.A.U.C.G.U.G.U.U.G.G.A.G.G.U.A.C.A.dT.dT | FMOD 3 |
| Ror1: AT GAA CCA ATG AAT AAC ATC | Sequence:<br>U.G.A.A.C.C.A.A.U.G.A.A.U.A.A.C.A.U.C.dT.dT<br>Complement:<br>G.A.U.G.U.U.A.U.U.C.A.U.U.G.G.U.U.C.A.dT.dT | ROR1-1 |
| Ror1: AAA AAT CTA TAA AGG CCA TCT | Sequence:<br>A.A.A.U.C.U.A.U.A.A.A.G.G.C.C.A.U.C.U.dT.dT<br>Complement:<br>A.G.A.U.G.G.C.C.U.U.U.A.U.A.G.A.U.U.U.dT.dT | ROR1-2 |
| Ror1: AC ATG TCA ATT CCA AAT CAT | Sequence:<br>C.A.U.G.U.C.A.A.U.U.C.C.A.A.A.U.C.A.U.dT.dT<br>Complement:<br>A.U.G.A.U.U.U.G.G.A.A.U.U.G.A.C.A.U.G.dT.dT | ROR1-3 |

Table 1B: Primer and probe sequences for RT-PCR

| Name | Sequence | Product length (bp) |
|---|---|---|
| FMOD-S | ACC GTC CCC GAT AGC TAC TT | 448 |
| FMOD-AS | CAT CCT GGA CCT TCC AGC AAA | |
| FMOD-Probe | Q-TCC AAC ACC TTC AAT TCC AGC AGG-9 | |
| Ror-1-S | CTG CTG CCC AAG AAA CAG AG | 545 |
| Ror-1-AS | CAT AGT GAA GGC AGCTGT GAT CT | |
| Ror1-Probe | Q-CAT GCT CAG CTG GTT GCT ATC AAG ACC -9 | |
| RPLP0-S | TTA AAC CCC CTC GTG GCA ATC | 297 |
| RPLP0-AS | CCA CAT TCC CCC GGA TAT GA | |
| RPLP0-Probe | Q-AGA TTG GCT ACC CAA CTG TTG CA-9 | |
| β-actin-S | CGA CAG GAT GCA GAA GGA GA | 161 |
| β-actin-AS | CGT CAT ACT CCT GCT TGC TG | |
| β-actin-Probe | Q-AAG ATC AAG ATC ATT GCT CCT CCT GAG-9 | |

Q= Blue-6-FAM 9= TAMRA FMOD= fibromodulin

Table 2: Volumes and concentrations of reagents used for siRNA transfection

| Culture Dish | 6-well plate | 24-well plate | 48-well plate |
|---|---|---|---|
| Complete growth medium | 800 µls | 250 µls | 200 µls |
| Opti-MEM® | 64 µls | 32 µls | 36 µls |
| TransIT-TKO® | 18 µls | 9 µls | 7 µls |
| 2 µM stock siRNA | 18 µls | 9 µls | 7 µls |

**Figure legends**

Supplemental Figure 1: Uptake of siRNA by CLL cells: CLL cells were transfected with green fluorescent siGLO® siRNA as described in Materials and Methods and examined at different intervals of time by flow cytometry. Suppl. Figure 1 A demonstrates the percentage of cells that were fluorescent compared to control, sham-transfected CLL cells. Each symbol represents cells of one patient Suppl. Figure 1B demonstrates the flow cytometry histograms of one representative patient's cells at various time intervals after transfection with siGLO® siRNA.

Figure 1: Downregulation of fibromodulin and Ror1 mRNA as examined by RT-PCR: CLL cells were transfected with siRNA against fibromodulin (FMOD) or Ror1 as described in Materials and Methods. Six hours later they were cultured on a monolayer of CD40L-transfected murine NIH 3T3 fibroblasts. After 24 hours, the CLL cells were collected and examined for expression of target mRNA by RT-PCR. Results of one representative experiment are shown. RPLP0 is a housekeeping gene that has been used as a control.

Figure 2: Relative quantitation of fibromodulin and Ror1 silencing by real-time PCR: CLL cells from 3 patients (black, grey and white bar, respectively) were transfected with siRNA and cultured as described in Figure 1. Downregulation of fibromodulin (FMOD) (A) and Ror1 (B) mRNA was examined by real-time PCR. The samples were subject to 35 cycles for fibromodulin and Ror1 and 30 cycles for the housekeeping keeping gene (β-actin). The expression of fibromodulin and Ror1 genes were normalized against the housekeeping gene as an internal standard. Gene expression by CLL cells transfected with control, non-silencing siRNA was regarded as 100% and gene expression of all other cells were expressed relative to it. Results of one representative experiment in each patient are depicted. The star denotes a sample in which fibromodulin mRNA could not be detected even after 40 cycles.

Figure 3 CLL cells treated with siRNA specific for fibromodulin (3A) or Ror1 (3B) show down regulation of the specific protein: CLL cells from three patients were transfected with FMOD-specific (3A) or Ror1-specific (3B) siRNA. 48 hours later, the cells were lysed and examined by Western blotting as described in Materials and Methods. In each case, lane 1 and lane 2 represent untransfected and control siRNA-transfected cells while lanes 3-5 represent CLL cells

treated with 3 different siRNA each against FMOD or Ror1. Beta actin used as a positive control in the experiments is demonstrated below each panel. Apoptosis of the cells following siRNA treatment was also examined and the results of the three patients are included in Figure 5.

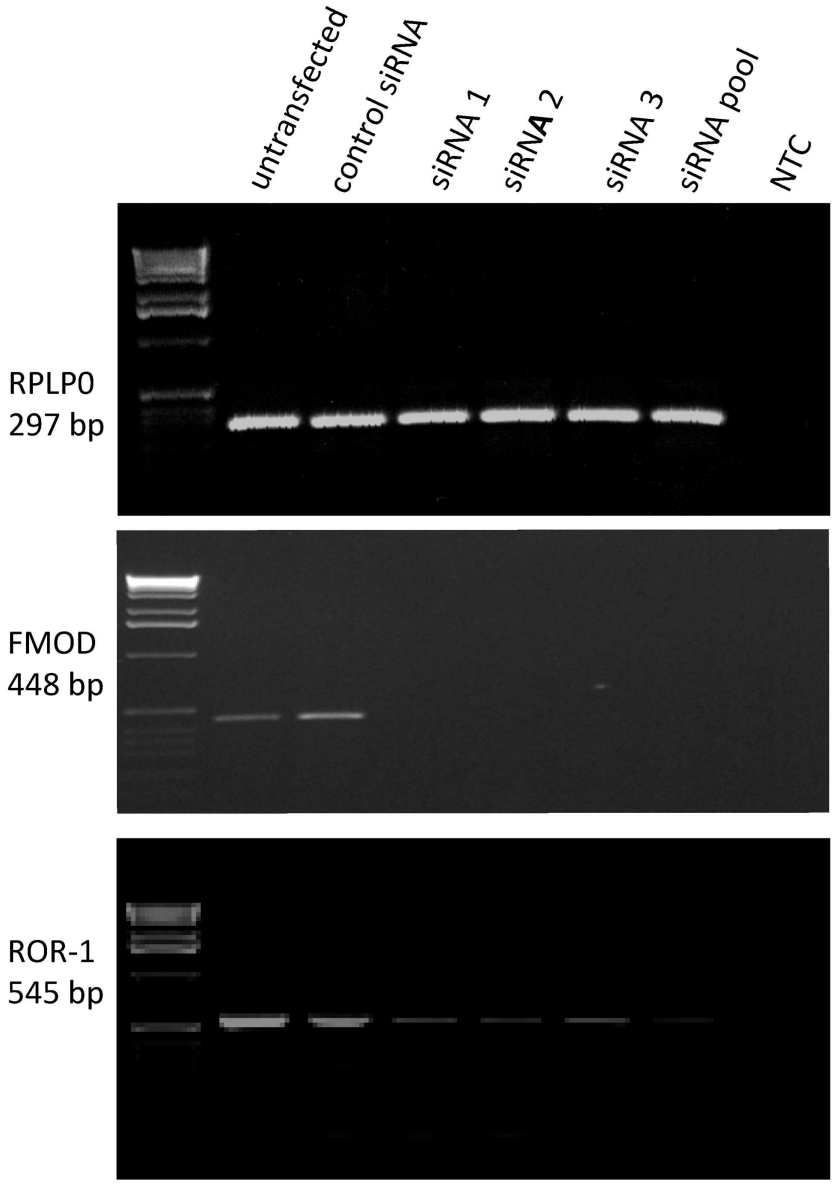
Figure 4: Changes in morphology of CLL cells following silencing of fibromodulin and Ror1: CLL cells were transfected with pools of three siRNAs against fibromodulin (FMOD) and Ror1 respectively. Six hours after transfection, the cells were transferred to monolayers of CD40L-transfected NIH 3T3 fibroblasts. Cells were visualized by light microscopy (Carl Zeiss Axiovert, Germany) after 24 hours. Results of a representative experiment are shown. A-untransfected; B- control siRNA-transfected; C-Cells transfected with pooled FMOD-specific siRNA; D- Cells transfected with pooled Ror1-specific siRNA.

Figure 5: Silencing of fibromodulin and Ror1 genes results in apoptosis of CLL cells: CLL cells were transfected with fibromodulin (A) and Ror1 (B) siRNA and examined for apoptosis by flow cytometry. Results with 6 patients are presented. Percentage of apoptotic cells (Annexin-V or Annexin-V/PI dual positive) is depicted on the Y-axis. The individual shaded bars each represent one patient's cells. The difference between each siRNA and untransfected cells or control siRNA was statistically significant ($p \leq 0.05$-$0.001$; Student's paired, two-tailed t-test).

Figure 6: Viability of peripheral blood cells from normal donors is not affected by siRNA against fibromodulin and Ror1: Peripheral blood mononuclear cells of normal donors were separated into a B cell-enriched and T cell-enriched fraction by nylon wool filtration. Cells were transfected with pools of three control, non-silencing siRNAs or pools of three siRNAs against fibromodulin (FMOD) and Ror1 respectively. Cells were labelled with anti-CD3 antibody to confirm enrichment into CD3+ and CD3- fractions, respectively prior staining with Annexin-V and propidium iodide. Results with 2 normal donors are presented. Percentage of apoptotic cells (Annexin-V or Annexin-V/PI dual positive) is depicted on the Y-axis.

Figure 7: Viability of human fibroblast cell lines after transfection with siRNA against fibromodulin and Ror1: Human fibroblast cells were transfected with pools of three control, non-silencing siRNAs or pools of three siRNAs against fibromodulin (FMOD) and Ror1 respectively. Cells were stained with Annexin-V and propidium iodide and analyzed by flow

cytometry. The experiment was repeated twice and mean values ± SD is presented. Percentage of viable cells (Annexin-V and PI negative) is depicted on the Y-axis.

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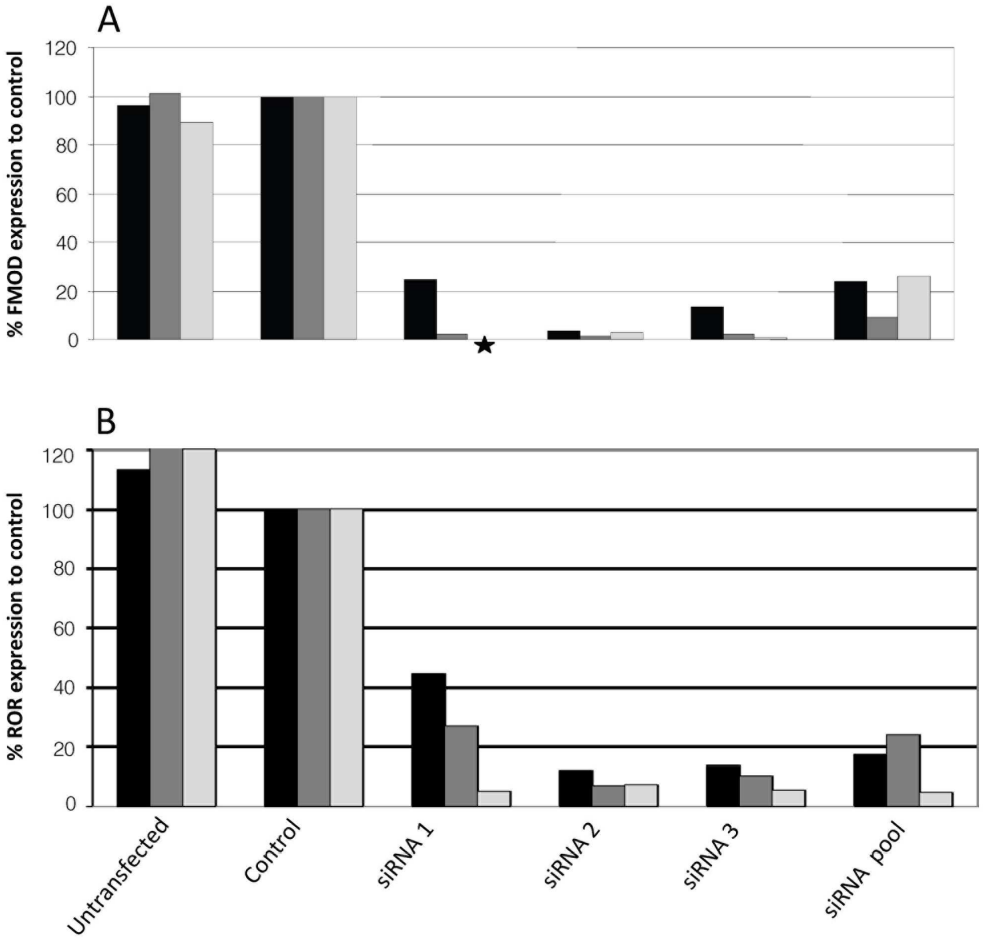


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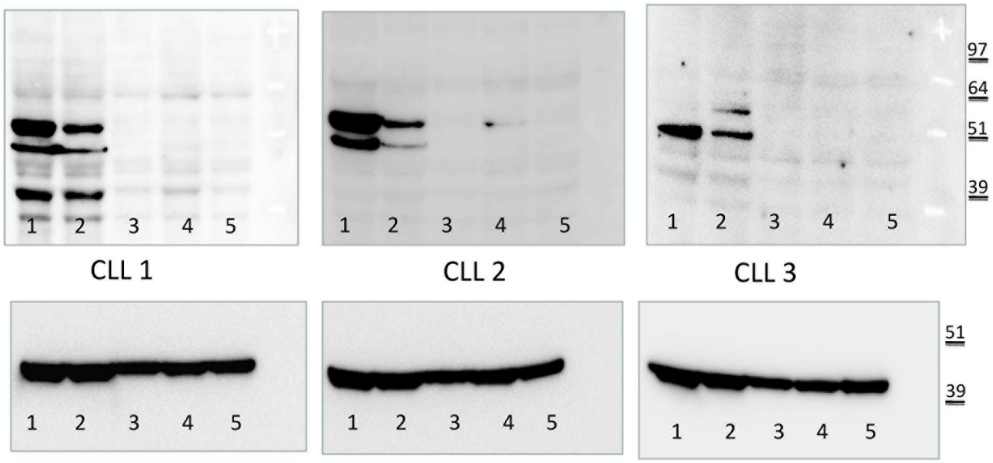


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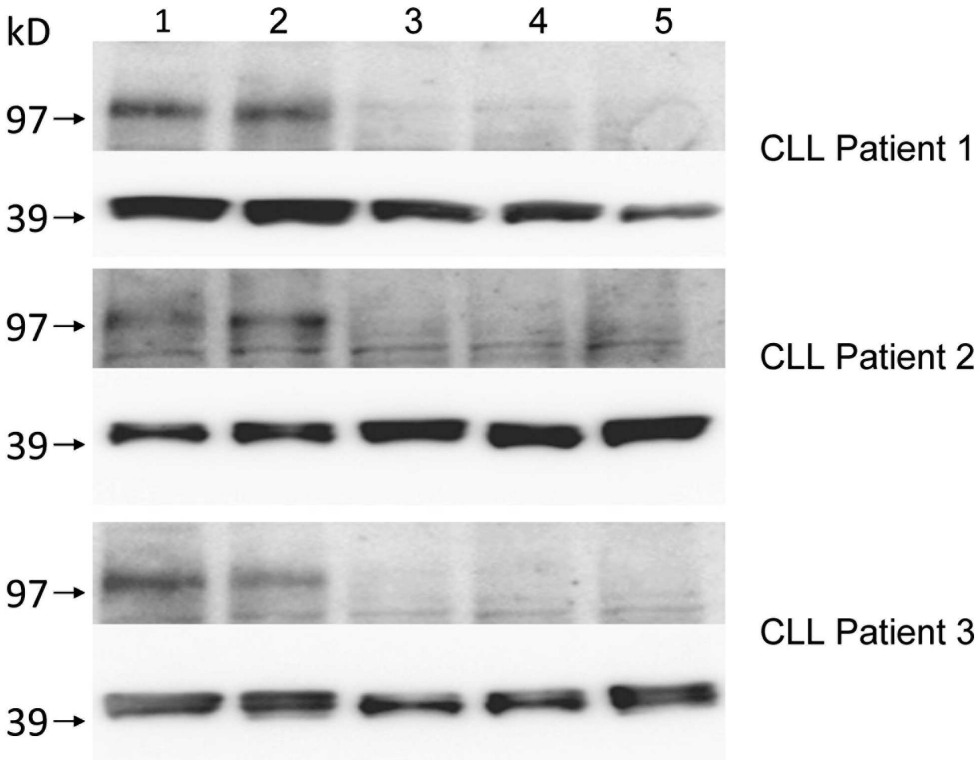


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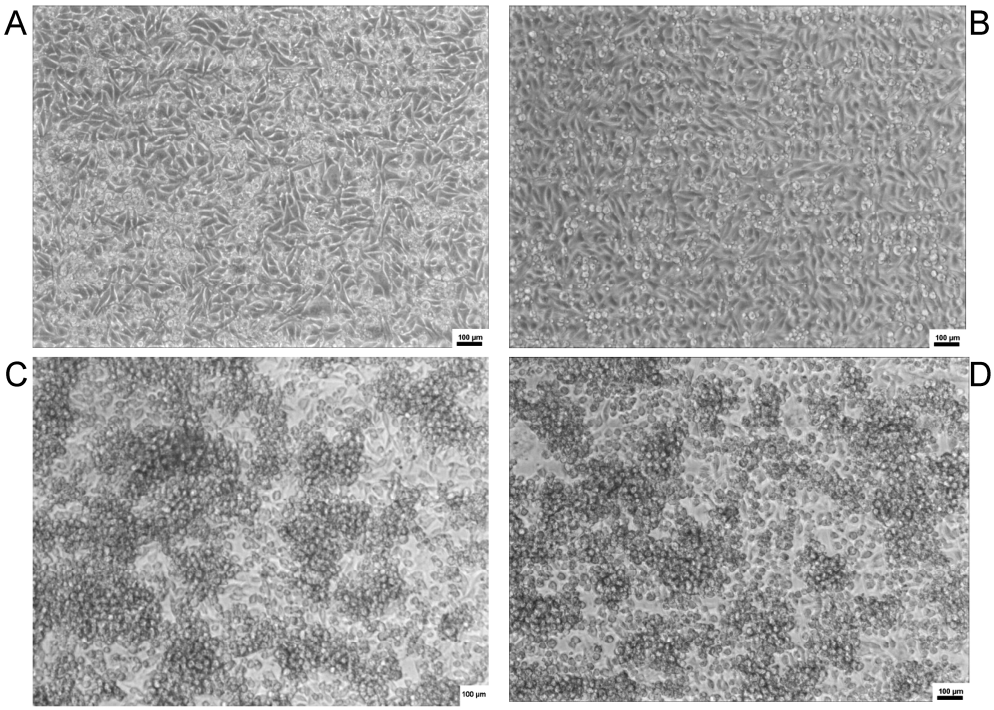


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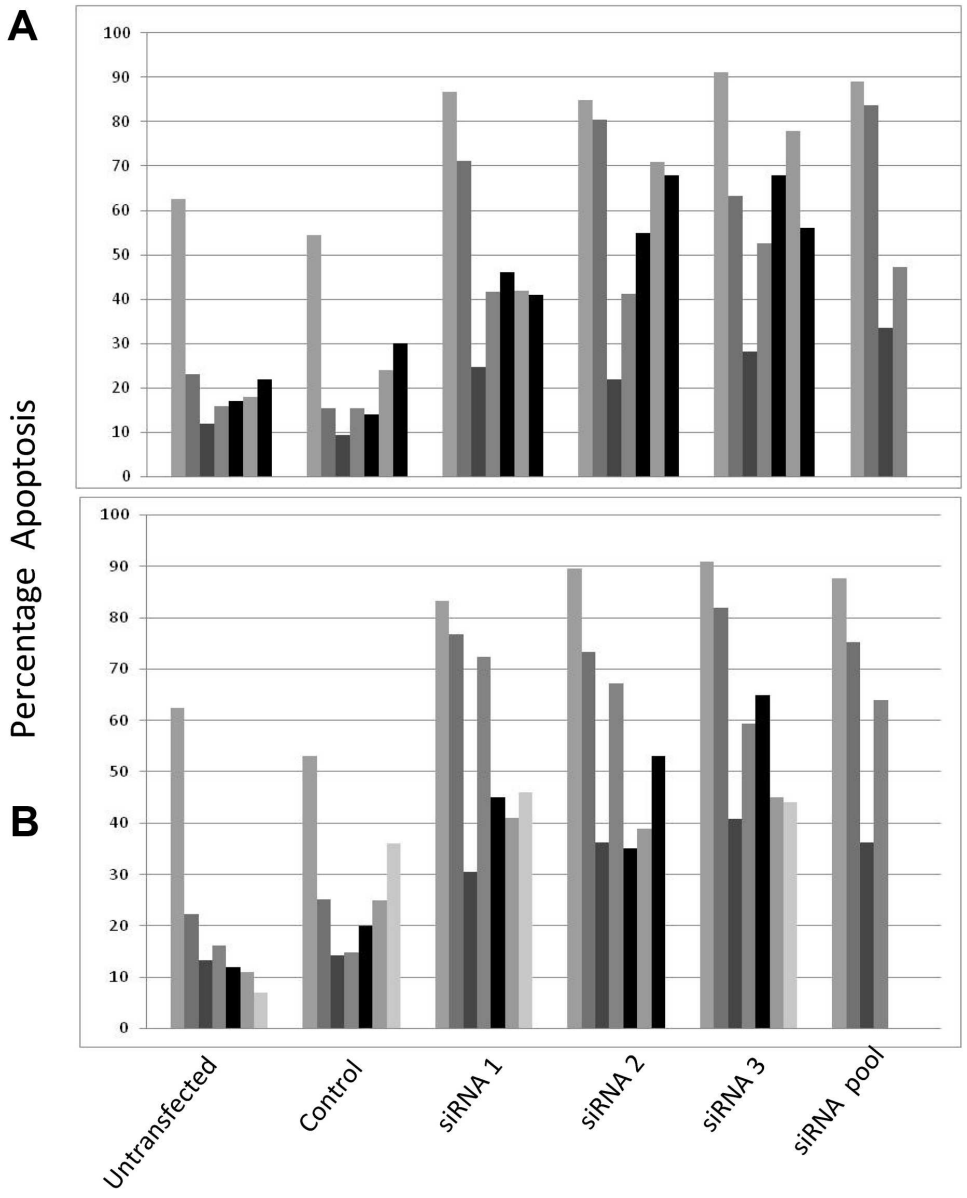


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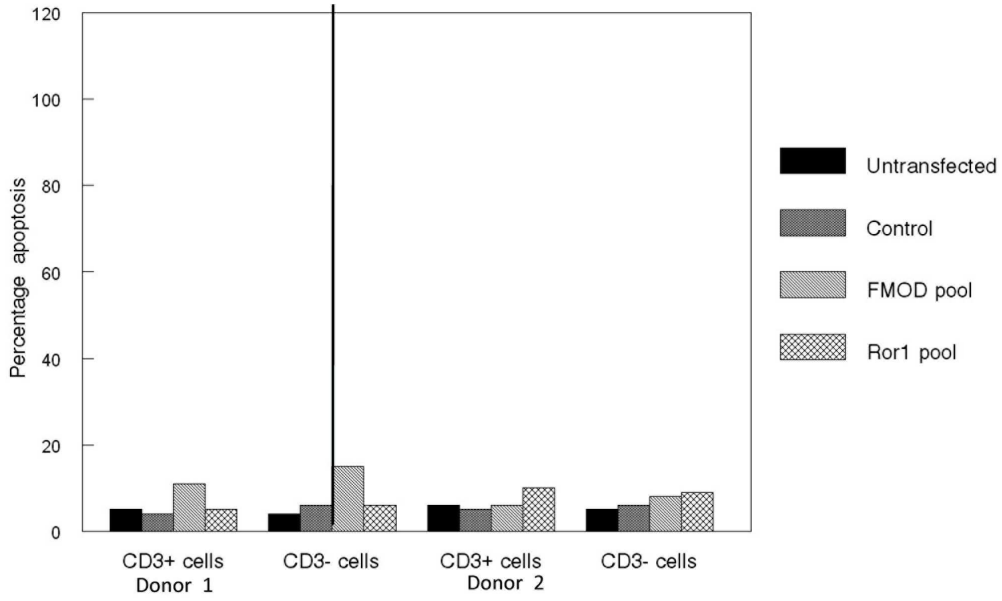


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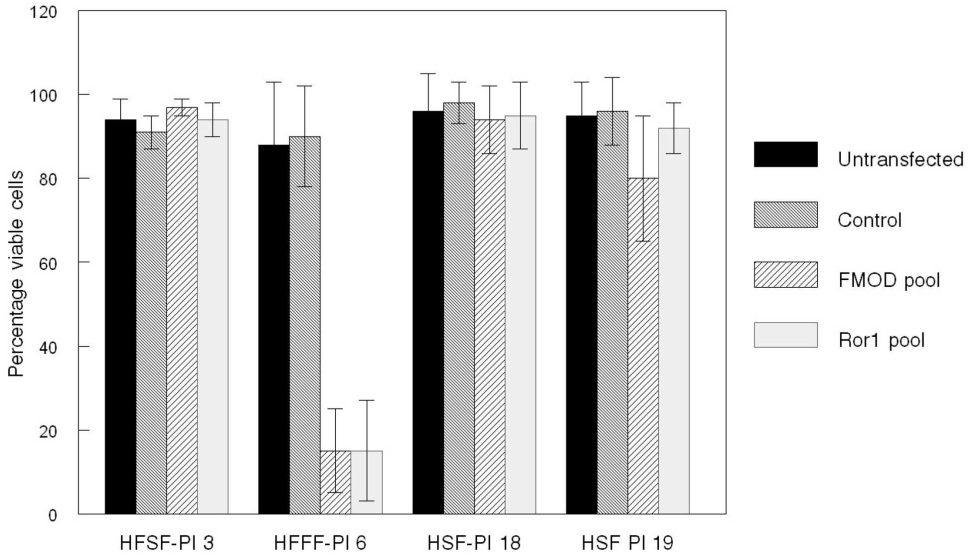


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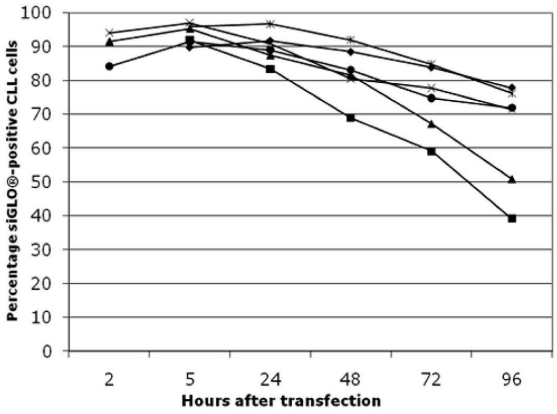
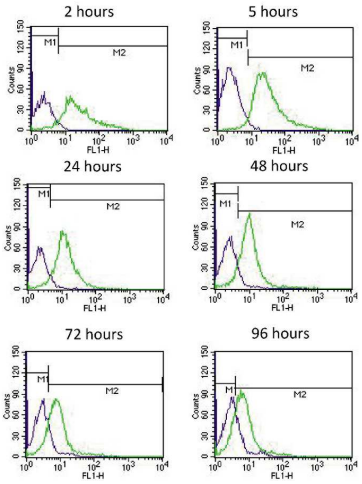



Supplementary Figur 1 A




Supplementary Figur 1 B

Suppl Fig 1A_1B. CLL cells were transfected with green fluorescent siGLO® siRNA as described in Materials and Methods and examined at different intervals of time by flow cytometry. Suppl. Figure 1 A demonstrates the percentage of cells that were fluorescent compared to control, sham-transfected CLL cells. Each symbol represents cells of one patient Suppl. Figure 1B demonstrates the flow cytometry histograms of one representative patient's cells at various time intervals after transfection with siGLO® siRNA.
160x89mm (600 x 600 DPI)