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Nonviral transfection of leukemic primary cells and cells lines by siRNA—a direct comparison between Nucleofection and Accell delivery

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Transient downregulation of genes in vitro employing short interfering RNA (siRNA) is a time-honored approach to study gene function. A crucial prerequisite to obtain a downregulation is an efficient and nontoxic delivery of the siRNA into the target cells. However, this has proven difficult to accomplish, particular in cells in suspension. Thus, there is a need for a systematic evaluation of different methodologies to identify the most suitable protocol. We compared Nucleofection with Accell, a novel nonviral-based delivery system in the setting of leukemic blasts from patients with myeloid leukemias. Two cell surface proteins, human inhibitory C-type lectin-like receptor and CD96, both believed to be associated with leukemic stem cells, were chosen as target genes. Accell not only yielded higher transfection rates, but also retained superior cell viabilities for both cell lines and primary leukemic cells. Thus, transfection efficiencies in primary cells after Accell delivery was 85% (range, 71–97%) compared to 38% (23–65%) using Nucleofection for siRNA delivery. Preliminary studies of clonal growth of primary acute myeloid leukemia cells indicated growth inhibition after siRNA transfection. Our results reveal that Accell delivery is suitable for nonviral transfection of cells in suspension, including primary leukemic cells. These data should provide a platform for further studies of genes involved in early leukemogenesis. © **2011 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

RNA interference (RNAi) is a powerful tool for investigating gene function in that it allows for gene silencing at the post-transcriptional level by introducing short interfering RNA (siRNA) complementary to the target messenger RNA into the cells of interest [1]. However, while effective in adherent cells, until now, published protocols have only to a limited extent proven efficient for cells in suspension, e.g., freshly isolated leukocytes or even continuous cell lines. The latter difficulty is of significant biological relevance because it is difficult to extrapolate information from cell line studies to the in vivo situation, e.g., for leukemia cells.

With the dual purpose of developing biologically relevant transfection protocols and studying the function of the human inhibitory C-type lectin-like receptor (hMICL) and CD96 proteins (see next section), we sought to knock down their corresponding messenger RNAs in primary acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) samples by means of RNAi as a model system for studying transfection in cells in suspension.

AML and CML are clonal myeloproliferative diseases of stem cell origin [2,3]. Despite high complete remission rates in patients with AML, nearly 80% of patients experience a relapse [4–6]. For CML, tyrosine kinase treatment has dramatically improved survival and >90% of patients can be expected to be alive and in molecular remission 5 years after diagnosis. It is generally recognized that a given leukemic cell population is biologically heterogeneous and consisting of still poorly defined leukemic stem cells as opposed to the mature leukemic blasts seen at diagnosis [7–9]. Recent studies have therefore attempted to identify molecular or genetic characteristics of the leukemic stem cells that would allow for a more specific identification of the leukemia-initiating clone. Here, the hMICL has been shown to be associated with myeloid leukemic stem cells [10,11], monocytes, granulocytes, and, to a lesser extent, dendritic cells [12–17]. This expression pattern implicates an important role of hMICL in hematopoiesis and potentially in myeloid leukemogenesis. Like hMICL, the CD96 protein

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has been shown to be associated with, and expressed on, the cell surface of malignant myeloid progenitor cells and not on progenitor cells of normal origin [18]. Prior studies on the functional role of these antigens in leukemia have employed xenograft transplantations of hMICL-positive stem cells, $CD34^+CD38$-$hMICL^+$ [10] and $CD90$-$CD96^+$ stem cell from AML patients into nonobese diabetic severe combined immunodeficient mice [18]. Another much less laborious approach could be the selective downregulation by siRNA, which has, moreover, entailed the potential of selectivity while retaining the integrity of the cell population.

The aim of this study was to identify the most effective nonviral method for siRNA delivery into AML and CML cell lines and primary cells. We report that the Accell method yields superior transfection rates in cells that are otherwise difficult to transfect by standard delivery methods. These data thus suggest that gene knock-down can now be attained in hitherto poorly transfectable myeloid leukemia samples.

Materials and methods

Primary leukemia cells

Mononuclear cells were isolated from peripheral blood (PB) and bone marrow (BM) by Lymphoprep (Nycomed, Oslo, Norway) and lysed by EasyLyse (DAKO, Glostrup, Denmark) according to manufacturers instructions. Three untreated AML patients and two untreated CML patients were included in these studies. For patient characteristics, see Table 1.

Cell culture

AML cell lines HL-60 and Kasumi-1 and the CML cell line K562 were purchased from Deutsche Sammlung von Mikrooganismen und Zellkulturen (DSMZ, Braunschweig, Germany). HL-60 and K562 are hMICL positive and were therefore included in knock-down validation of hMICL. Kasumi-1 is CD96-positive and was included in CD96 knockdown experiments. All cell lines were maintained in exponential growth phase in RPMI supplemented with 10% heat inactivated fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cell lines were kept at 37°C in a fully humidified atmosphere of 5% CO_2 in air.

siRNA and transfections

Three chemically synthesized anti-hMICL siRNAs, three anti-CD96 siRNAs, and one fluorescein (FITC) labeled anti-Cyclophilin B siRNA (designed and synthesized by Dharmacon, Lafayette, CO, USA) were transfected into both cell lines and primary mononuclear cells using Accell delivery media (Dharmacon), according to manufacturer's instructions and using serum-free conditions (https://www.thermo.com/eThermo/CMA/PDFs/Various/File_4814.pdf). An FITC-labeled nontargeting scrambled siRNA was used as control in each sample. Table 2 lists sequences of anti-hMICL and anti-CD96 siRNA.

For Accell delivery, a cell concentration of 25×10^3 and 50×10^3 cells/100 μL delivery media was used for the cell lines and primary cells, respectively. An siRNA concentration of 1 μM siRNA/100 μL delivery media was used in all reactions. Nucleofection was performed using the Amaxa Nucleofector and the Cell Line Nucleofector Kit V and Kit T (Lonza, Cologne, Germany) according to manufacturer's instructions. Based on preliminary studies programs P-13, T-02, and T-16 were used for K562 and programs T-01, S-11, and T-19 for HL-60. Nucleofector Kit V (Lonza) was used for both cell lines. Primary AML cells were nucleofected using the Nucleofector Kit T and the program U-15. Primary CML cells were nucleofected using the Nucleofector Kit V and the program T-20 [19]. A concentration of 3 μg siRNA was used in all reactions.

Viability and transfection efficiencies

Necrosis and apoptosis post-Nucleofection and Accell transfection was determined by flow cytometry using the Annexin V-PE Apoptosis Detection Kit I (BD, Franklin Lakes, NJ, USA). Transfection efficiencies on live cells were determined by flow cytometry by determining FITC-positive cells after transfection using FITC-labeled siRNA.

Real-time quantitative PCR (qRT-PCR)

Detection of knockdown levels was measured on a MX3000 Pro (AH Diagnostics, Aarhus, Denmark) using TaqMan qRT-PCR. All qRT-PCR reactions were performed in triplicate and data normalized to the mean of the two housekeeping genes *β_2-microglobulin* (*β2M*) and *abelson* (*ABL*) found to be the most stable control genes in hematopoietic cells by the Europe Against Cancer group [20]. Primers and probes targeting hMICL, CD96, and Cyclophilin B were designed and purchased from Applied Biosystems (Carlsbad, CA, USA). Primer and probes for the two housekeeping genes *β2M* and *ABL* were purchased from DNA Technology (Aarhus, Denmark).

Statistics

Data are presented as mean percentages with range. Student's *t*-test was used to compare transfection efficiencies after Nucleofection and Accell transfection with p values < 0.05 deemed statistically significant.

Table 1. Patient characteristics

Patient no.	Sex	Age	Diagnosis	Leukocytes at diagnosis (10^6/mL)	% hMICL at diagnosis	% CD96 at diagnosis
1 (AML #1)	M	47	AML	67.7 (PB)	56	42
2 (AML #2)	M	79	AML	17.5 (PB)/66.2 (BM)	75/79	58/67
3 (CML #1)	F	53	CML	41.7 (PB)	43	65
4 (CML #2)	F	44	CML	45.8 (PB)	69	27
5 (AML #3)	M	18	AML	185 (PB)	89	57

hMICL = human inhibitory C-type lectin like receptor.
Cells from CML patients were all taken from diagnosis in stabile phase.