

Complex of small non-coding RNAs piR-30074 and antago-miR-155 and miR-125b with DDMC carrier transforms Girardi heart cells into CD4+ cells.

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Abstract. Girardi heart cells are cell culture of right atrial cells contaminated with HeLa cervical adenocarcinoma cells. The combined cells have genotypic characteristics of cervical cancer. In this study, was investigated influence of complex of DDMC vector with small non-coding RNAs (sncRNAs) on Girardi Heart cells. As the result, the genotypic and phenotypic characteristics of Girardi heart cells were changed. Micro-RNAs (miRs) and pi-RNAs (piRs) are sncRNAs, a class of small epigenetic regulatory molecules that control normal development and differentiation of different cell types. For transfection, the cells were treated with a complex of non-viral DDMC vector with piR-30074 and antago-miR-155, followed by the addition of a complex of DDMC vector with miR-125b. I observed OCT4 gene and protein expression in the cells after treatment using the complexes of DDMC vector with piR-30074 and antago-miR-155. The expression of KIR2DL1, GITR1, DICER1, PIWIL1, RISC1, FLT3, NOS2, MAFB, LIN28 genes was detected and the expression of CD4+ and CD117+ protein markers was observed. The expression of these genes and proteins was not observed in control cells.

Keywords: Girardi heart cancer cells, antago-miR-155, piR-30074, miR-125b, DDMC vector, transformation

Introduction: Small non-coding RNAs (sncRNAs) are a respectable family of non-protein coding regulatory RNAs that modify the genetic program of cells. Some RNAs act in the nucleus, while others regulate extra-nuclear processes. A combination of nuclear and extra-nuclear regulating

sncRNAs can be used to fully reprogram any type of cells. I previously used different sncRNAs to reprogram cancer cells. Treatment of Kasumi-1 leukemic cells with antago-miR-155 transformed acute myeloid leukemia cells into platelet-like cells[1]. MiR-155 and miR-125b are immune-miRs and regulate lymphopoiesis from hematopoietic stem cell [2, 3]. In this study, were used two sncRNAs and one mimic of miR-155 for reprogramming of Girardi heart (GH) cervical adenocarcinoma cells into OCT4+ cells as intermediate form and into CD4+, CD117+ cells as the final form.

Materials and Methods:

Cells: Girardi heart (ECACC® 93120822™)(HeLa derivative) is human cervical adenocarcinoma cell line and is one of types was found to be indistinguishable from HeLa by STR PCR DNA profiling. Therefore, the cell line should be considered as derived from HeLa was proceeded in accordance with the ECACC protocol. Cells were thawed and routinely maintained in accordance with the standard method. I incubated cells in Dulbecco's Modified Eagle Medium (DMEM), containing 10% Fetal Bovine Serum, 2 mM l-glutamine, 100 mg/ml penicillin, and 100 ME/ml streptomycin (GE Healthcare) and incubation parameters at 37 °C with 5% CO₂. After 2 days cells were prepared for further cultivation in in the growth concentration of cells 0,5x10⁶/ml. After accumulation of cells number 1x10⁶ / ml, cells were prepared to transfection with nanoparticles.

Choose of sncRNAs for treatment: In the preliminary investigations were used bioinformatics tools to choose most reliable candidates from small non-coding RNAs families for Girardi Heart cells treatments. Small RNA targets are predicted mainly by three computational algorithms: 1) Lewis BP, and co.; 2) Krek A, and co.; 3) Griffiths-Jones S, and co. [4-6].

In the series of pilot experiments with GH cells were selected more than 20 snc-RNAs. Finally, only two sncRNAs sequences were used for further investigations. The third sequence was previously chosen in studies with Kasumi-1 cells [1]. Oligonucleotide sequences were for antago-miR-155 (antisense for miR-155 (MIMAT0004658)): 5'- GAG GAU GUA UAA UCG UAA UUG

U-3', and for piR-30074 (DQ569962.1): 5' – AAAGCTTTAAGTGTGTTGGCGTGCTTC – 3', for miR-125b (MIMAT0004592): 5' – ACGGGUUAGGCUCUUGGGAGCU – 3'.

Carriers:The cationic graft-copolymer (**DDMC Vector®** (Non-viral Transfection reagent) from Ryuju science co., Aichi, Japan was purchased by SID ALEX GROUP, Ltd. (Prague, Czech Republic).

Manipulations with cells: The solution of the DDMC Vector was warmed to 37°C. Solutions for transfection were prepared as follows. In a sterile tube, 10 µg of each snc-RNAs was diluted to 990 µl in medium and added 8 µl of the cationic graft-copolymer. The concentration of the DDMC vector was 8µl/ml of medium and sncRNAs 10 µg/ml of medium. The procedure of cells treatment was described in previous study [7]. Cells were incubated with complexes of DDMC vector with piR-30074 and antago-miR-155 for 21 days and then they were analyzed. One part of cells removed in the 21st day was treated with RNAeasy mini kit (Qiagen, USA) and used for isolation of total RNA, reverse transcription reaction and specific cDNA transcript amplification. Another part of cells was stained using the Leishman-Romanowsky method [8]. The third part of cells was used for detection of OCT4+ protein expression by immunofluorescence method. In the fourth part of cells, I added complex of DDMC vector with miR-125b and incubated for 14 days. One part of these cells was used for isolation of total RNA, reverse transcription reaction and specific cDNA transcript amplification. Another part of cells was stained using the Leishman-Romanowsky method. The third part of cells was used for detection of OCT4+, CD4+, and CD117+ proteins expression (Santa Cruz Biotechnology, Inc., USA; R&D, USA). Cells were stained with a FITC staining reagent (R&D, USA) and were photographed under fluorescent microscopy (AxioVertA1, Zeiss, Germany).

For the control were used cells without any treatment, and cells, which were treated with unloaded DDMC vector. All manipulations with cell cultures were repeated for three times.

Gene expression analysis: Total RNA was extracted from cell culture using the RNAeasy Mini kit (Qiagen, USA) according to the manufacturer's protocol. In these series of experiments a two-

step reverse transcriptase-PCR standard procedure was used. All products were from Invitrogen Co. Amplification of CASP8, PIWIL1, ICOS1B, HMOX1, CKIT1B, KIR2DL1, DICER1, NOS2, RISC1, OCT4, KI67, BCL11B, TGFBR2, THBS1, and beta-actin cDNA (as an internal control) was performed with an automatic thermocycler (TProfessional, Biometra, Germany). The primers sequences 5'-3' were:

- 1) CASP8 NG_029188.1 5'-TCCAGATTGACGACAAGTGC -3', 5'-CACTCAGGAAGACGCGTTAC -3';
- 2) PIWIL1 ENST00002452555'-TGCTATTCACCGGCTTCCTT-3', 5'-TGCTCACTCCTGAAAGTACGT-3';
- 3) ICOS1B OTTHUM00000256369 5'-GCTTTGAAGCATCTCCCTTG-3', 5'-ACGTGTGCTTTTACCCCAAG-3';
- 4) HMOX1 ENST00000216117 5'-ACATCTATGTGGCCCTGGAG -3', 5'-TGTTGGGGAAGGTGAAGAAG -3';
- 5) CKIT1B ENST00000288135 5'-GACTCATGGGCTTGGGAATA-3', 5'-ACTTCAGGGGCACTTCATTG-3';
- 6) KIR2DL1 ENST00000611849 5'-CTGGAATCTGAAGGCGTGA-3', 5'-GGCAGGGGTCAAGTGAAAT-3';
- 7) DICER1 ENST000000526495 5'-GCCCCGTTAATTATGCTTGA -3', 5'-ACTCGCACAGAGGCATTTCT -3';
- 8) NOS2 ENST00000313735 5'-ACATCCCCGCAAACATAGAG-3', 5'-TACCAGGAGGAGATGCTGGA-3';
- 9) RISC1 (AGO2) ENST00000220592 5'-GCGCACCATGTACTTTGCT-3';
- 10) OCT4 ENSG00000204531 5'-GTAGAGACGGGGTTTCACCA-3', 5'-CAGAGCATCGTGAAAGGACA-3';
- 11) KI67 5'-TGCAAACAGGTCAGGAAGG-3', 5'-CTGCCCCCAAGTTCTTGAT-3';
- 12) BCL11B ENST00000345514 5'-TGGTAAGCTGGTGAGCACTG-3', 5'-GGAGGGAATGGGAGAGAAAG-3';
- 13) TGFBR2 ENST00000295754 5'-GCAACAGCTATTGGGATGGT-3', 5'-TTTGATGGTGGGAAGGTCTC-3';
- 14) THBS1 ENST00000366787 5'-TGATGGATAGGGGGCAAAT-3', 5'-CTTCGTTGGTCTCGGGAAT-3';
- 15) MAFB ENST00000373313.25'-CCAGCCTTGACCTGTTTGAC -3', 5'-CTTGGTGACTTCTCGGGACT -3'.

PCR products were loaded on 2 % agarose gel and electrophoresed then colored with Ethidium Bromide, exposed to a gel doc system (Syngene, India) and quantified using Quantity One Software

(Bio-Rad, Germany). For comparison inner control – expression of beta-actin gene, and external control for which were used GH cells without any treatments. Sequences for inner control beta-actin (ENST00000331789) gene primers were: 5'-TCCCTGGAGAAGAGCTACGA-3', 5'-AGCACTGTGTTGGCGTACAG-3'.

Statistics. All gene expression data were normalized to internal control gene expression levels of beta-actin. For external control were used culture of cells without any treatment in the same moment of time as experimental cells. All samples were prepared in triplets. Experiments were repeated three times (N=9). AltAnalyze software was used for genes expression analysis. Data are presented as the mean \pm SEM (observed differences between control and experimental cells were considered statistically significant if p-values were $P = .05$).

Results: In the following experiments, the newDDMC Vector from Ryuju science co. was used for all transfection procedures. Twenty-one days after the complex of DDMC vector with sncRNAs was added, GH cells exhibited genetic and morphological changes compared to non-treated control cells. The expression of the DICER1, RISC1, and PIWIL1 genes was observed in the transfected cells (Fig.1). The expression of these genes are expressed then stimulated sncRNAs/PIWI/AGO cascade. The OCT4 gene was also expressed in transfected cells. I observed the expression of OCT-4 protein in cells by immunofluorescent microscopy (Fig. 2.E.). The expression of the MAFB and LIN28 genes was increased compared with control cells. Polymorphism of the MAFB gene was observed in control cells but not the experimental group. Increased expression of the HMOX1, CKIT1B, KIR2DL1, ICOS1B, TGFBR2, NOS2 and THBS1 genes was detected in transfected cells compared with control cells. I also obtained morphological changes in the transfected cells. The cells formed large, homogeneously stained spheres with light-stained cells were on the periphery of the spheres. The sphere expressed the OCT4 protein, as detected by immunofluorescence (Fig. 2.B., 2.E.). It was supposed, that the cells composing the spheres had pluripotent properties. After treatment, using a complex of DDMC vector with miR-125b, was observed further transformation of the cells. On the 14th day after transfection using a complex of DDMC vector and miR-125b, the cells were present as

separate cells. The cells were smaller in size than in the control group and had one sharp nucleus inside each cell (Fig. 2.C.). The CD4 marker was detected on the cell surface by immunofluorescence (Fig. 2.F.).

Figure 1. Heat maps of the 17 differentially expressed genes found in two independent microarray studies in GH cancer cells after treatment with complexes of DDMC vector with piR-30074 and a-miR-155, and subsequent treatment with complexes of DDMC vector with miR-125b to compare with control cells. The heat map was produced by hierarchical clustering of the probeset data. Probesets for genes are represented by columns with the gene dendrogram at the top. Blue color indicates decreased expression and Red color indicates increased expression of genes (AltAnalyze Platform).

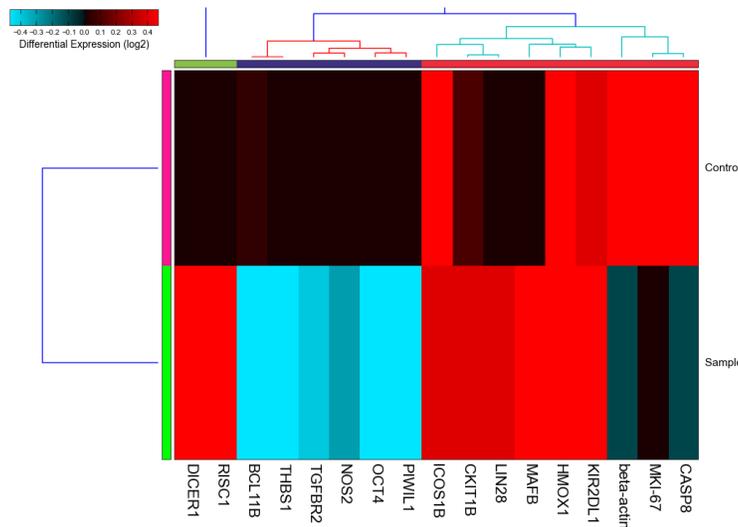
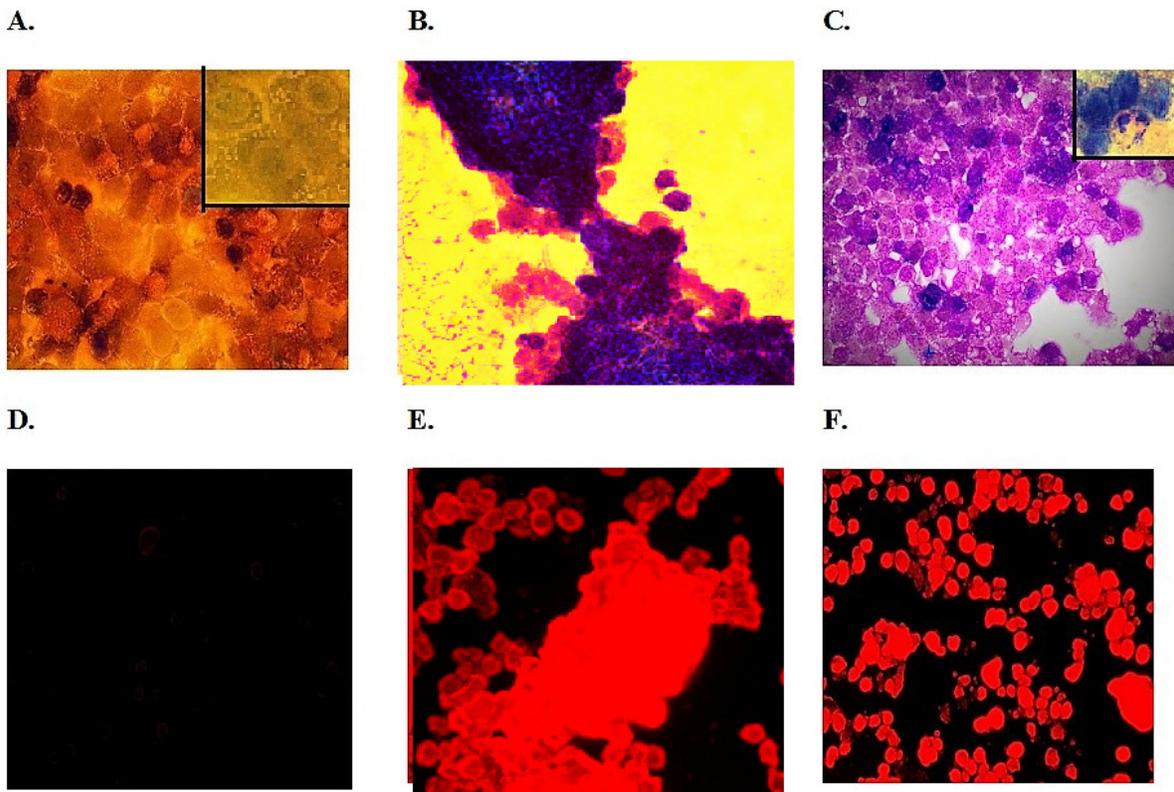


Figure 2. Microscopic photos of GH cells, stained using the Leishman-Romanowsky method: A. Control group of cells (Magnification x600); typical GH cancer cells (photoenhanced at the right top corner, cell diameter is app. 30 μm). B. Transformed cells 21 days after

treatment using complexes of DDMC vector with piR-30074 and antago-miR-155 (Magnification x600). C. Cells 21 days after treatment using complexes of DDMC vector with piR-30074 and antago-miR-155, and 14 days after adding of complexes of DDMC vector with miR-125b. (Magnification x600); four CD4 expressed cells (photo enhanced at the left top corner)(cell diameter is app. 6 μ m).

Fluorescent microscopy of GH cells: D. Control cells after staining using CD4+/FITC antibodies. E. Transformed cells 21 days after treatment using complexes of DDMC vector with piR-30074 and antago-miR-155, stained using CD117+/FITC antibodies (Magnification x600). F. Cells 21 days after treatment using complexes of DDMC vector with piR-30074 and antago-miR-155, and 14 days after adding of complexes of DDMC vector with miR-125b(staining using CD4+/FITC antibodies)(Magnification x600).



In the control GH cells, the expression of the ICOS1B, CKIT1B and KIR2DL1 genes was decreased. However, high expression of these genes was observed in transfected cells. These genes

were expressed in CD4+ cells [9-11]. The expression level of HMOX1 was increased more than four-fold compared with the controls. CKIT1B gene expression was increased more than five-fold compared with the control. The SOX2, LIN28 and KLF4 genes were not expressed in the control and experimental groups. Other genes were also investigated, but did not exhibit a response.

In these experiments, CD4 was used as a phenotypic marker of lymphocytes, and OCT4 was used as a marker of pluripotency [12]. The transformed cells expressed the CD4 and OCT4 markers, which were detected by fluorescence microscopy.

Discussion:

In this study, I observed genetic and morphologic changes in GH cells 21 days after treatment with a complex of DDMC vector as carrier and antago-miR-155 and piR-30074 and 14 days after the addition of a complex of DDMC vector and miR-125b. On the 21st day, the cells were fully transformed into a transitory form. In this study, **were used only sncRNAs** for the transformation and further differentiation of cells. The intermediate form of cells formed 21 days after transfection using the complex of DDMC vector with antago-miR-155 and piR-30074 and expressed the OCT4 gene and protein; however, expression of the CD117 and CD4 proteins was not observed in these cells. On the 14th day after treatment of the cells using the complex of DDMC vector with miR-125b, the transitory form of the cells transformed into separate sharp mononuclear cells that expressed the CD4 and CD117 markers. The intermediate form obtained from GH cancer cells expressed the marker OCT4. Interestingly, OCT4 is a key factor in maintaining and re-establishing the pluripotent state of cells. This state is transient *in vivo*, and embryonic stem cells can maintain pluripotency indefinitely *in vitro*. Previous studies identified micro-RNAs that up- or down-regulate OCT4 gene and protein expression in embryonic stem cells to potentiate the reprogramming of embryonic stem cells to primitive mesoderms or trophoblasts. Treatment with antago-miR-155 promoted the expression of OCT4, which reprograms embryonic stem cells into the mesoderm stage. The reprogramming of cells in the early stage is achieved by four defined transcription factors, the Yamanaka factors (Oct4, Sox2, Klf4

and c-Myc) or the Thomson factors (Oct4, Sox2, Nanog and Lin28), both of which include Oct4. Each cell type expresses one or several reprogramming factors and require fewer factors for reprogramming[13-16].sncRNAs alone or in combination have been shown to reprogram human and mouse cells to iPSCs without requiring exogenously expressed transcription factors [17-19].The targets of these sncRNAs are involved in many processes that are required for iPSC reprogramming or pluripotent cell maintenance, including cell proliferation, inhibition of chromatin remodeling factors, regulation of apoptosis, and, potentially, regulation of alternative splicing factors. piR may support the actions of selected micro-RNAs and cause genetic changes in cancer cells during the reprogramming processes. The main function of piR is the regulation of transposable elements (mostly Alu elements), which provide a vast repertoire of homologous sequences scattered throughout the genome that can be involved in non-allelic homologous recombination leading to deletions and duplications that result in cancer. Components of transposable elements, including both transcripts and proteins, are elevated in cancers compared with normal tissues. The Piwi protein is a typical suppressor of position effect variegation, similar to other key epigenetic factors such as heterochromatin protein 1 (HP1a), and interacts with other key epigenetic factors. Piwi deficiency results in global loss of methylation of histone 3 at lysine 9 (H3K9me), the delocalization of HP1 from polytene chromosomes, and loss of euchromatic features at a sub-telomeric region of chromosome 3R. Piwi and its homologs are key components of an epigenetic regulatory complex required for euchromatin/heterochromatin assembly [20-24]. These features indicate that treatment with piRs is necessary for the complete genetic transformation of cells. Interestingly, the exact combination of miRNAs varies among different studies, and these different combinations might have varying reprogramming efficiencies, or the combination required might be cell-type specific. This variation complicates the selection of sncRNAs variants for reprogramming. Different cell types have specific transcriptomes and probably require the repression of different genes by sncRNAs to allow initiation of reprogramming. Studies of transcription factor-mediated reprogramming have indicated that a number of steps are involved in the transformation of a somatic cell into a pluripotent cell [25, 26]. In this study we detected increased levels of the MAFB

gene in the experimental cells. Expression of this gene is strongly connected with the action of the piR/PIWI system. MAFB is part of piR/PIWI regulatory circuit and also acts as a repressor of the self-renewal enhancers: Myc, KLF2, and KLF4 [27, 28].

Studies from the past several years have clearly established an important role of miRNAs in T cell development, differentiation into the appropriate subsets, establishment of immunological memory and the maintenance of homeostasis in the periphery. In the next series of experiments, I observed morphologic changes in the cells and expression of the CD4 marker after treatment with a complex of DDMC vector and miR-125b. The development of T lymphocytes into functional cells is characterized by the expression of T cell receptors. CD4/CD8 double-negative cells are the early T cell progenitors that differentiate into CD4/CD8 double-positive cells and then CD4 or CD8 single-positive cells. Upon Dicer1 deletion a significant decrease in CD4 T cells was observed in the spleen and lymph nodes. These CD4 T lymphocytes exhibited increased levels of apoptosis, defective proliferation and aberrant cytokine production, indicating that miRNAs are essential for the proper maturation and function of T lymphocytes in the periphery. MiR-21, cluster miR-17—92, miR-155, miR-146, and miR-181, among others, are immune-miRs that regulate hematopoiesis and T cell differentiation and function by controlling different pathways [25, 26, 29-34]. MiR-125b expression may enhance naïve CD4+ T cell development by enhancing specific genes and pathways. MiR-155 is also an immune-miR that regulates the functions of hematopoietic stem cells [29-34].

All of these genetic and morphological changes are due to cell death or irreversible transformation. As a result, structural modification of cancer tissues occurs.

Treatments using long-lived DDMC vector complexes with molecular epigenetic regulators such as sncRNAs would be un-complicated and cost-effective due to the slow biodegradation of nanoparticles, thus making this an attractive method for the development of new and effective anti-cancer drugs.

Conclusion: Adding of the slowly-biodegradable complex of non-viral DDMC vector with piR-30074 and antago-miR-155, and consequent adding of DDMC vector with miR-125b in GH cancer cells results in transformation of GH cancer cells into CD4+ cells.

The author declare no conflict of interest.

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