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Can the reprogrammed cancer cells serve as an alternative source of (induced) cancer stem cells?

Ewelina Stelcer[1], Małgorzata Blatkiewicz[1], Karol Jopek[1], Wiktoria Maria Suchorska[2,3], Marcin Rucinski[1]

[1]Department of Histology and Embryology, Poznan University of Medical Sciences, Poznan, Poland
[2]Department of Electroradiology, Poznan University of Medical Sciences, Poznan, Poland
[3]Radiobiology Lab, Greater Poland Cancer Centre, Poznan, Poland

ABSTRACT

Background: Cancer stem cells (CSCs) constitute a small and elusive subpopulation of cancer cells within a tumor mass and are characterized by stem cell properties. Reprogrammed CSCs exhibit similar capability to initiate tumor growth, metastasis, and chemo- and radio-resistance and have similar gene profiles to primary CSCs. However, the efficiency of cancer cell reprogramming remained relatively low. There is limited literature available on the reprogramming of lung cancer cells. Hence, in this study we have conducted reprogramming of human lung cancer cells towards more benign type of cells.

Materials and methods: The reprogramming process was carried out with the use of STEMCCA vector. We have investigated the gene expression profile of induced CSCs (iCSCs) using the microarray technique.

Results: The lung iCSCs demonstrate morphology characteristics of induced pluripotent stem cells (iPSCs) and gene expression profile that significantly differ from cells before reprogramming. We have also presented the elevated level of expression of genes associated with the cancer stemness and thus revealed new interesting CSC-like molecular markers.

Conclusions: These preliminary results demonstrated that the reprogramming process *in vitro* leads to the remarkable changes in cells at the gene level and has potential to be an alternative method of generating CSC-like cells.

Key words: cancer stem cells; non-small cell lung cancer; reprogramming; gene expression profile

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Introduction

Non-small-cell lung cancer (NSCLC) constitutes a heterogeneous group of malignancies. For patients with NSCLC stages I through IIIA surgery is the first treatment of choice. Patients with resected lung cancer are characterized by a high risk of relapse. Therefore, they are treated with adjuvant chemotherapy. In turn, patients with stage IIIB and IV NSCLC are routinely treated with chemotherapy with the option of surgery [1–3]. Although the diagnosis and treatment of NSCLC become more and more advanced, lung cancer patients demonstrate high rates of recurrence, metastasis, and resistance to applied therapy [4]. The lung cancer stem cells (CSCs) constitute a small subset of undifferentiated cells which are involved in the development of lung cancer progression. The identification of lung CSC biomarkers is nowadays of great interest [5]. It is well known that benign cells can acquire cancer phenotype after a malignant transition, but whether cancer

Address for correspondence: Małgorzata Blatkiewicz, Department of Histology and Embryology, Poznan University of Medical Sciences, Poland; e-mail: mblatkiewicz@ump.edu.pl

cells can be genetically and epigenetically reversed back to a benign state is not fully explored [6, 7]. Induced CSC (iCSC) technology and therapy may be a breakthrough in the prevention of cancer generation and progression. Accordingly, cancer cells that have been reprogrammed *via* the introduction of a single or several genes, which are capable of triggering a SC phenotype, can be a good model of several aspects of cancer research [8]. This approach involves the study of cancer heterogeneity and niches, the elucidation of the mechanisms of cancer initiation and progression, epigenetic reprogramming, screening of compounds as therapeutic or re-differentiating agents, and induction of cell death/senescence for cancer ablation therapy [9]. Thus, the aim of this study was to carry out a reprogramming process on the human lung cancer line and investigate the gene expression profile of generated iCSC.

We hypothesize that reprogramming cancer cells leads to generation of CSC-like cells that can serve as a base for a reliable 3D cancer model. Those iCSCs, in contrast to CSCs isolated from tumor, could serve as a potentially unlimited source of patient-specific CSCs. The iCSCs could be used in a wide variety of 3D model-based research concerning the influence of CSCs on disease course and response to implemented therapy. Although this concept constitutes an emerging field, we believe that the reprogramming process is an ideal candidate for creating a 3D cancer model that is possibly closest to the clinical picture.

Materials and methods

Study was performed using NSLC (NCI-1299, ATCC) cell line. That cell line was cultured according to instructions available on the official ATCC web site: RPMI 1640 medium (Biowest, France), buffered with 1.134 g/L NaHCO3 and 14.5 mM HEPES (Bio&Sell, Germany). Medium was supplemented with 10% FBS (Biowest, France).

We performed a reprogramming process with the use of STEMCCA Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit (Merck Millipore, Germany) on NSLC (NCI-1299, ATCC) cell line that according to ATCC is a suitable transfection host and its response to the *in vitro* reprogramming process has not yet been described in the literature.

For iCSCs generation from NCI-1299 cell line the procedure was as follows: 1 × 10 4 NCI-1299 cells at passage 3 were seeded on a 6-well plate in a complete appropriate culture medium according to manufacturer's instructions. Cells were transduced twice with STEMCCA-tetO lentiviral vector (MOI = 50) in the presence of 5 μg/mL Polybrene (Sigma Aldrich, MO, USA). Cells were cultured in 3 ml of complete medium for six days until they reached 90% confluence. Then, lentiviral transduced cells were replated on Matrigel™ Matrix (Corning, MA, USA) coated 6-well plates with Mitomycin C (Sigma Aldrich, MO, USA) inactivated mouse embryonic fibroblasts (MEF) feeder layer. Cells were detached with 1 ml of ACCUMAX™ (Merck, Germany), counted and 1.5–2 × 10 4 cells were plated on a well of a 6-well plate and cultured in 3 mL of hiPS medium (DMEM/F12, 20% Knockout Serum Replacement, 1 × non-essential amino acids, 1 × β-mercaptoethanol, 0.5% penicillin/streptomycin and 10 ng/ml of basic Fibroblast Growth Factor (bFGF) (all from Thermo Fisher Scientific, MA, USA) in standard culture conditions (37°C, 5% CO2). The medium was changed every other day. Twenty-one days after the first transduction, most resembling iPSC colonies (with even edges and with numerous tiny cells of the same size) were manually picked up and transferred to a new 6-well plate (one colony per well) coated with Matrigel™ Matrix (Corning, MA) and a MEF feeder layer.

The gene expression profile of obtained iCSCs were studied using Affymetrix Human Gene 2.1 ST array strips according to previously established protocols [10–12]. Briefly, the analysis involved tabular data that included details about fold change, adjusted p-value, and normalized counts for each comparison. This data was analyzed using a BioConductor repository with the statistical programming language R (version 4.1.2; R Core Team 2021). To identify differentially expressed genes (DEGs), certain criteria were applied: an absolute fold change greater than two and a p-value with a false discovery rate (FDR) correction below 0.05. The outcomes of this selection were visualized through volcano plots, which depicted the total count of up-regulated and down-regulated genes. The DEGs obtained from each comparison were further analyzed using the DAVID bioinformatics tool, which stands for Database for Annotation,

Visualization, and Integrated Discovery. The gene symbols of the DEGs were uploaded to DAVID using the "RDAVIDWebService" BioConductor library. Subsequently, the significantly enriched Gene Ontology (GO) terms from the GO BP Direct database were identified. To ensure the reliability of the results, the p-values of the selected GO terms were corrected using the Benjamini–Hochberg correction method.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) method was used to confirm microarray- based results. The primers were designed using Primer 3 software (Whitehead Institute for Biomedical Research, MA, USA). The expression level for each target gene was calculated as $-2\Delta\Delta Ct$.

To confirm the viability of created spheroids from iCSC, we performed EthD-1 staining (Thermo Fisher Scientific, MA, USA).

Results

The reprogrammed cells revealed morphology of iPSCs and have the ability to form live spheroids (Fig. 1A). Experiments were performed on Affymetrix Human Gene 2.1 ST array strips. All bioinformatic analyses was carried out using Bioconductor package that is a component of the biostatistic programming R language. For background correction, normalization and summation of raw data, the Robust Multiarray Averaging (RMA) algorithm implemented in "affy"

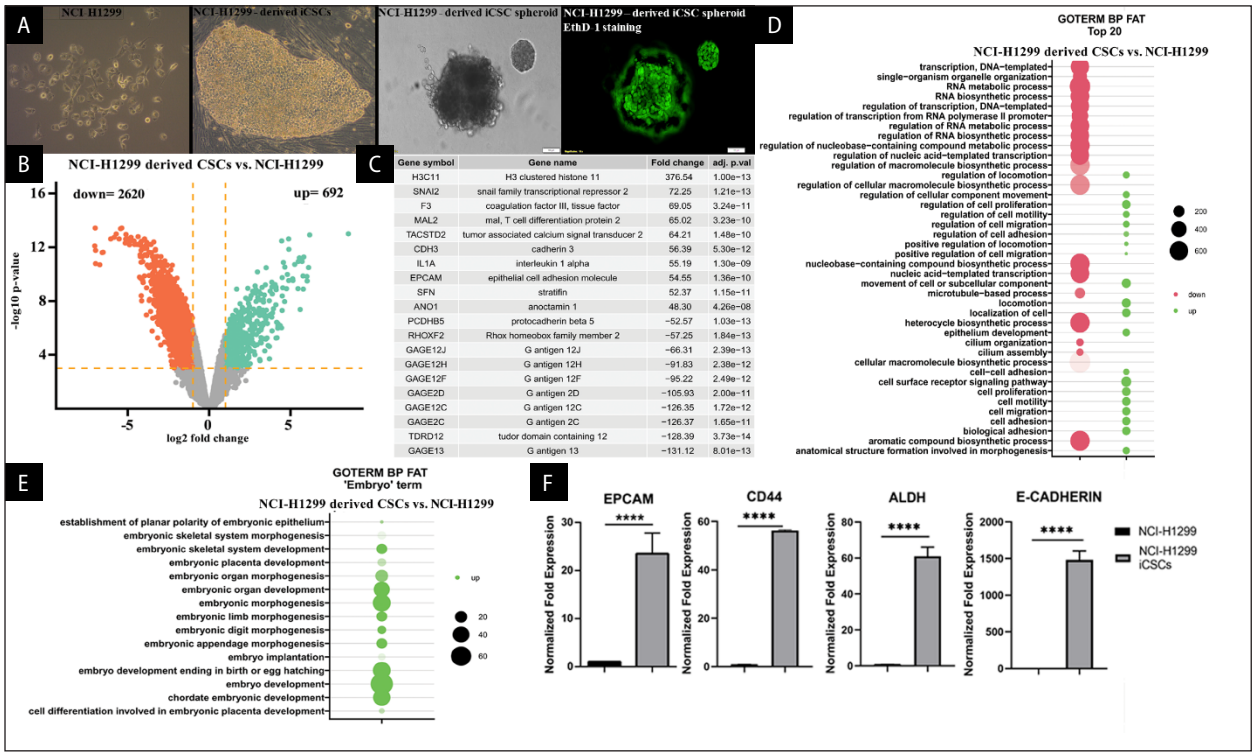

Figure 1. NCI-1299 cell line after reprogramming had the ability to form iPSC-like colonies as well as create live spheroids (**A**). The selection criteria for significant changes in gene expression were based on absolute fold-change > 2.0 and p-value with false discovery rate (FDR) correction (adjusted p-value) of 0.001. The result of this selection was presented as a volcano plot, showing the total number of upregulated and downregulated genes affected by reprogramming process (down = 2620 and up = 692) (**B**). The top 10 upregulated (e.g. H3C11 = 376.54) and downregulated genes (e.g. GAGE13 = −131.12) are presented in table (**C**). All gene IDs of differentially expressed genes (DEGs) were uploaded to DAVID with the use of the RDAVIDWebService Bioconductor library, where they were assigned to relevant Gene Ontology (GO) terms, with a subsequent selection of significantly enriched GO terms from the GO BP FAT database. It involves, among others, the following biological processes: "RNA metabolic process", "cell motility" and "cell adhesion" (fold-change > 2.0 and adjusted p-value < 0.001) (**D**) and those processes are engaged in early development like: "embryo development", and "embryonic morphogenesis" (fold-change >2.0 and adjusted p-value < 0.05) (**E**). Finally, the more benign status was also confirmed by qRT-PCR analysis. It involves the increase of following gene expression markers: *EPCAM*, *CD44*, *ALDH*, *E-CADHERIN*)

package of BioConductor, was applied. Biological annotation was taken from BioConductor "oligo" package, where annotated data frame object was merged with normalized data set, leading to a complete gene data table. The general profile of transcriptome changes is shown as a volcano plot. We assumed the following selection criteria: an expression |fold difference| (absolute value) > 2 and $p \leq 0.001$ (Fig. 1B) to generate a number of genes or biological processes that could be presented and described in a clear scientific way. The result of such a selection was presented as follows: the top 10 upregulated and downregulated genes after the reprogramming process are presented in the table in Fig. 1C. To determine the biological processes most likely to be regulated by the reprogramming process, we analyzed enrichment in the relevant ontological groups from the GO BP database (Fig. 1DE). The acquired stemness status was confirmed by qRT-PCR (*EPCAM*, *CD44*, *ALDH*, *E-CADHERIN*) (Fig. 1F).

Discussion

Cancer stem cells (CSCs) play an important role in the development of lung cancer. CSCs are resistant to drug and radiation therapies and are responsible for tumor recurrence. Therefore, other research methods targeting CSCs are urgently needed [13]. The success in reprogramming a somatic cell into a stem-like (human induced pluripotent stem cells, hiPSCs) state has led to the idea of reprogramming malignant cells back to their less differentiated state [14]. Such iCSCs appear to have a CSC-like state after the reprogramming process. The generation of iPSC-like cells from cancer cells (induced cancer stem cells, iCSCs) may provide tools for exploring the mechanisms of tumor initiation and progression *in vitro*, for studying the plasticity of cancer cells and for achieving cancer type specific drug discovery [15]. There are several major obstacles in cancer cell reprogramming to overcome before iCSCs can be widely used, including improvement of reprogramming efficiency and, particularly, the lack of characterization of iCSC-derived 3D *in vitro* models [16]. Through the Yamanaka factor-mediated reprogramming, varied types of cancer cells, including leukemia, breast, bladder, liver, prostate, and pancreatic cancer cells, were stably reprogrammed into CSCs. So far, the reprogramming process using cell lines derived from lung cancer is very poorly described in the literature [17].

Several groups have reported the reprogramming of cancer cells including melanoma, pancreatic cancer, gastrointestinal cancer, bladder cancer, lung carcinoma, and breast cancer. The cancer-derived iPSCs were distinct from parental cancer cells in tumorigenic activities [18, 19].

Mathieu and co-workers (2011) reported that reprogramming factors and hypoxia-inducible factor 1 alpha accelerated the induction of iPSCs from the A549 lung carcinoma cell line. In addition, the reprogrammed pancreatic, liver, and colorectal cancer cells were able to differentiate into various lineages, including epithelial, mesenchymal, and neuronal cells [20].

Bang and collaborators (2019) were unable to obtain iPSCs from MCF10A and MCF7 breast cancer cell lines. Consequently, they identified 29 candidate barrier genes based on RNA-sequencing data [21]. Chao et al. (2017) generated iPSCs from acute myeloid leukemia (AML) patient samples. AML iPSCs lacked leukemic potential, but when differentiated into hematopoietic cells, they reacquired the ability to give rise to leukemia *in vivo* and reestablished leukemic DNA methylation/gene expression patterns [22].

The reprogramming methods remain a challenge because of two issues: the cancer-specific epigenetic state and the chromosomal aberrations or genetic mutations present in cancer cells [23]. To effectively manipulate such epigenetic lesions to induce lung cancer cell reprogramming, it is necessary to obtain a comprehensive understanding of the reprogramming mechanisms from a molecular perspective [24].

Our study has a major limitation: our research was performed with the use of only one cell line — NCI-1299. Nevertheless, we obtained very promising and high-throughput-based method preliminary results and, hence, we decided to publish them as a brief report. In the near future, markers that were selected by our group should be verified using more comprehensive methods and in a larger context. Especially, pluripotency genes should be taken into account.

Conclusions

Our preliminary result proved that the reprogramming process *in vitro* may be a promising alternative to generating cancer cells with more benign characteristics. We also demonstrated new molecular markers characteristic of those benign cells.

Moving forward, these preliminary results pave the way for further exploration into the implications of reprogramming *in vitro*. Beyond elucidating the mechanisms underlying CSC generation, our study suggests that reprogramming holds promise as an alternative approach for generating CSC-like cells. Future research endeavors could delve into refining reprogramming techniques, elucidating the functional consequences of altered gene expression, and exploring the therapeutic implications of targeting CSC-like populations.

More detailed experiments should answer the following questions:

- what is the most suitable and reproducible reprogramming system for generating iCSCs from human lung cancer cells?;
- how similar/different are 3D structures formed from non-reprogrammed and from reprogrammed lung cancer cells;
- what is the tumorigenic potential of multi-cellular 3D structures generated from lung cancer cells and iCSCs? *iv)* how do those 3D structures respond to applied therapy and, consequently, could they be an appropriate future drug screening platform? And finally: is the reprogramming process a better way to create a successful 3D model for lung cancer research in comparison with established and primary lung cancer cell lines? We strongly believe that our or other group will soon provide answers to the above key questions.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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