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Reduced Expression of *PEDF* and *ALDH1A1* during Spheroid Transition of Lung Cancer Cells: An In Vitro Study

M. Y. Terzi[a, b, *, **] (ORCID: 0000-0001-8478-0451), H. M. Okuyan[c, d], G. Gülbol-Duran[a, b], and M. Urhan-Küçük[a, b]

[a] *Department of Medical Biology, Faculty of Medicine, Hatay Mustafa Kemal University, Hatay, Turkey*
[b] *Department of Molecular Biochemistry and Genetics, Graduate School of Health Sciences, Hatay Mustafa Kemal University, Hatay, Turkey*
[c] *Department of Medical Services and Techniques, Vocational School of Health Services, Hatay Mustafa Kemal University, Hatay, Turkey*
[d] *Department of Physiotherapy and Rehabilitation, Faculty of Health Sciences, Sakarya University of Applied Sciences, Sakarya, Turkey*
**e-mail: menderesyusufterzi@gmail.com*
***e-mail: myterzi@mku.edu.tr*

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Abstract—"Cancer stem cells" (CSCs) can initiate tumorigenesis and metastasis and show resistance against chemotherapy owing to the expression of prominent stem cell markers. CSCs are a subpopulation of highly heterogenic cancer spheroid cells. Pigment epithelium-derived factor (PEDF) is a neurotrophic, anti-tumorigenic, and anti-metastatic protein and its gene expression levels in A549 spheroids is still unknown. We aimed to compare clonogenicity and mRNA levels of *PEDF*, *Oct4*, and *ALDH1A1* between A549 and spheroid cells. Spheroid and colony formation assays were performed with spheroid and A549 cells. We performed quantitative reverse transcription-polymerase chain reaction (qRT-PCR) for gene expression analysis. The clonogenic ratios for A549 and spheroids were ~60% and ~1% respectively. During spheroid formation, *Oct4* mRNA level did not change but *PEDF* and *ALDH1A1* levels decreased significantly. CSCs are characterized by elevated stem cell markers but spheroid cells consist of heterogeneous population including CSCs. In spheroid population, no increase in stem cell markers was observed. The reduced *PEDF* levels during spheroid transition can be a suppression mechanism of spheroid cells.

Keywords: lung cancer, *PEDF*, A549, spheroid cell, *Oct4*, *ALDH1A1*

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INTRODUCTION

The term "cancer stem cells" (CSCs) was first used to define "circulating tumor cells" (CTCs) which can express stem cell markers together with metastatic and high tumorigenic features (Jordan, 2004; Ucar, 2009; Massague, 2016). These cells are characterized with self-renewal, sustainable proliferative potential, and the differentiation capacity into tumor types originated from three germ-layers (Bonnet, 1997). Thus CSCs were also identified in various solid tumor types (lung, breast, and colon cancers, osteosarcoma, melanoma, etc.) among the heterogeneous tumor cell population (Duru, 2014; Lee, 2016). CSCs can resist chemotherapies owing to augmented expression levels of drug transporter proteins (Wang, 2013).

Spheroid cells are utilized to enrich CSCs in vitro. Unlike other cancer cells, spheroid cells can survive and proliferate under serum-free-medium supplemented with certain growth factors (EGF, bFGF, etc.) in ultra-low binding conditions (Allegra, 2014; Xu, 2018). In vitro-cultivated cancer spheroid cells are highly tumorigenic and metastatic owing to its stem cell-like features. Liu et al. previously showed that after culturing gastric cancer cell line in special stem cell conditions, the cancer spheroids gained CSC-like features such as self-renewal, higher proliferation, drug resistance, and tumorigenicity (Liu, 2013). Furthermore, the transplantation of cancer spheroid cells were previously shown to form tumors in nude mice with a remarkably higher tumorigenicity than the adherent cancer cells (Adhikari, 2010; Spyra, 2011; Morrison, 2012). Besides, spheroids can be enriched by using drug-conditioned media or identified and sorted with flow cytometry using specific markers such as CD133, ALDH1A1, Oct4, etc. (Chen, 2008; Eramo, 2008; Eyler, 2008; Wang, 2013). It should be also taken into consideration when working with cancer spheroids that there is highly heterogeneous cell population within cancer spheroids derived from

either cell lines or primary tumor tissues (Cheng, 2016; Herreros-Pomares, 2019; Pang, 2019).

Pigment epithelium-derived factor (PEDF), also known as serpin family F member 1, is a non-inhibitory member of serine protease inhibitor family and a versatile protein characterized as anti-tumorigenic, anti-metastatic, anti-angiogenic, neurotrophic, and neuroprotective (Bouck, 2002; Fernandez-Garcia, 2007; Minkevich, 2010). PEDF is expressed in almost all fetal and mature tissues however its levels decrease by aging and malignancy (Belkacemi, 2016). In a clinical study performed with 91 NSCLC tissues, the overall *PEDF* gene expression was shown to decrease significantly compared to normal tissues (Zhang, 2006). Together with its anti-apoptotic and proliferative effects on the cells after tissue injuries/degenerations (Terzi, 2015), anti-tumorigenic effects of PEDF have been shown against several cancer types with many in vivo and in vitro studies (Demestre, 2013; Wang, 2013; Hong, 2014). In another clinical study conducted with breast cancer patients, upregulated PEDF levels were found to be correlated with a better prognosis (Zhou, 2016). So far, the expression pattern of *PEDF* as a prominent anti-tumorigenic protein has not been demonstrated during the transition to spheroid cells from adherent A549 cells. In this study, we aimed to analyze the gene expression levels of *PEDF* together with putative CSC markers, i.e., *Oct4* and *ALDH1A1*, in cancer spheroids originated from parental A549 human adenocarcinoma cell line.

MATERIALS AND METHODS

Cell Culture of A549 Cell Line and Spheroid Cells

We purchased A549 cell line, as a non-small cell lung cancer (NSCLC) in vitro model, from the American Type Culture Collection (ATCC). The cell line was cultured in DMEM (Gibco, UK) with 10% FBS (Gibco, Brazil) and 1% penicillin/streptomycin and cultured in an incubator at 5% CO_2 and 37°C. A549 adherent cells were called as "parental cells" in the following sections of the text and used as the control group for further experiments.

We produced spheroid cells by sub-culturing parental A549 adherent cancer cells in ultra-low binding plates/flasks (Corning, Maine, USA) containing serum-free DMEM, 5 ng/mL human basic fibroblast growth factor (bFGF, Life Technologies, Maryland, USA), 10 ng/mL human epidermal growth factor (EGF, Invitrogen, California, USA), and 1% B27-Supplement (Life Technologies, New York, USA). Spheroid cells were passaged at day 7 by supplying fresh medium at every 4th day. After second passage, the spheroids were used for further experiments.

Spheroid and Colony Formation Assays

Spheroid and colony formation assays were performed with spheroids and A549 cells respectively to analyze clonogenic efficiency. We performed spheroid formation assay as described previously (Roudi, 2014). Shortly, 3.5×10^3 cells/mL were seeded into 6-well ultra-low binding plates for 11 days. The spheroids ($\varnothing > 80$ μm) were counted with a phase contrast microscope (Nikon Eclipse TS100, Tokyo, Japan). Colony formation assay was conducted based on Roudi et al. with minor modifications (Roudi, 2014). Briefly, 80 cells per well were seeded into 6-well plate and after 5 days of incubation, the colonies consisting of 10–15 cells were counted with microscopy for analysis. The colony/spheroid formation efficiency was calculated as the percentage of initial seeded number of cells. The data were presented as mean ± *SD*.

RNA Isolation and qRT-PCR

We performed quantitative reverse transcription-PCR (qRT-PCR) for gene expression analysis after total RNA isolation. The parental and spheroid cells were seeded into 6-well plates at a density of 10^5 cell/well. After confluency of A549 and after 7 day-incubation for spheroids, total RNA was isolated (Genejet RNA Purification Kit, Thermo Fisher, USA) following cDNA synthesis using 2 μg RNA (High Capacity cDNA RT Kit, Thermo Fisher Scientific, Lithuania) in a thermal cycler. Diluted cDNA samples were used for qPCR analysis of POU class 5 homeobox 1 (*Oct4*), aldehyde dehydrogenase 1 family member A1 (*ALDH1A1*), serpin family F member 1 (*PEDF*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as housekeeping for normalization using Maxima SYBR Green detection method (Thermo Fisher Scientific, Lithuania) with Rotor Gene Q (Qiagen, Hilden, Germany) with the following cycling conditions: 50°C for 2 min, 95°C for 10 min, 40 cycles of; 95°C 15 s, and 60°C for 1 min. The list and specifications of designed primers (Thermo Fisher Scientific, Lithuania) for each gene was shown in Table 1. The fold changes were calculated based on $2^{-\Delta\Delta Ct}$ method and the data were presented as mean ± *SEM*.

Statistical Analysis

All experiments were performed at least in triplicates. Data analyses of gene expression assays were performed by using "RT^2 Profiler PCR Data Analysis" (Qiagen, online service) based on $2^{-\Delta\Delta Ct}$ method. The gene expression results were expressed as "fold change" compared to the control group. The statistical difference between two groups was determined with student's *t*-test. $p < 0.05$ was considered as significant for statistical comparisons.

Table 1. Designed primers for qRT-PCR

Gene	Primer sequence	Amplicon (bp)	Reaction conditions
Oct4-F	5'-TTCAGCCAAACGACCATCT-3'	142	60°C, 40 X
Oct4-R	5'-GGGTTTCTGCTTTGCATATCTC-3'		
ALDH1A1-F	5'-GCCATAACAATCTCCTCTGCTC-3'	128	60°C, 40 X
ALDH1A1-R	5'-CCGTACTCTCCCAGTTCTCTT-3'		
PEDF-F	5'-AGATGAAGCTGCAATCCTTGT-3'	112	60°C, 40 X
PEDF-R	5'-CCATCCTCGTTCCACTCAAAG-3'		
GAPDH-F	5'-GTCAACGGATTTGGTCGTATTG-3'	106	60°C, 40 X
GAPDH-R	5'-TGTAGTTGAGGTCAATGAAGGG-3'		

Oct4: POU class 5 homeobox 1, *ALDH1A1*: Aldehyde dehydrogenase 1 family member A1, *PEDF*: Serpin family F member 1, *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase, bp: Base pair, F: Forward primer sequence, R: Reverse primer sequence, Reaction conditions (Annealing temperature, X: Times of cycle).

RESULTS

Spheroid and Colony Formation Assays

A549 and spheroid cells were depicted in Figs. 1a–1c. Spheroid cells were obtained and photographed at day 7 after sub-culturing parental A549 cells under low attachment and serum-free conditions (Fig. 1a). We performed spheroid formation assay to analyze colony formation efficiency (CFE) of the spheroid cells. As depicted in Fig. 1c, the size of spheroids increased by the incubation time (Fig. 1c). Then the total number of spheroids that are larger than 80 μm in diameter was counted in each well. And it was found that after 11 days incubation time, CFE of the spheroid cells was ~1% (Fig. 1d).

Then we also checked clonogenicity of A549 parental cells. As depicted in Fig. 1b, after 5 days of incubation, the total number of colonies consisting of more than 10–15 cells were counted per well and CFE was calculated as ~60% (Fig. 1d).

Gene Expression Levels of PEDF, ALDH1A1, and Oct4

In the second part, we measured the gene expression levels of target genes in spheroids and A549 cells. We showed after analysis that *PEDF* and *ALDH1A1*

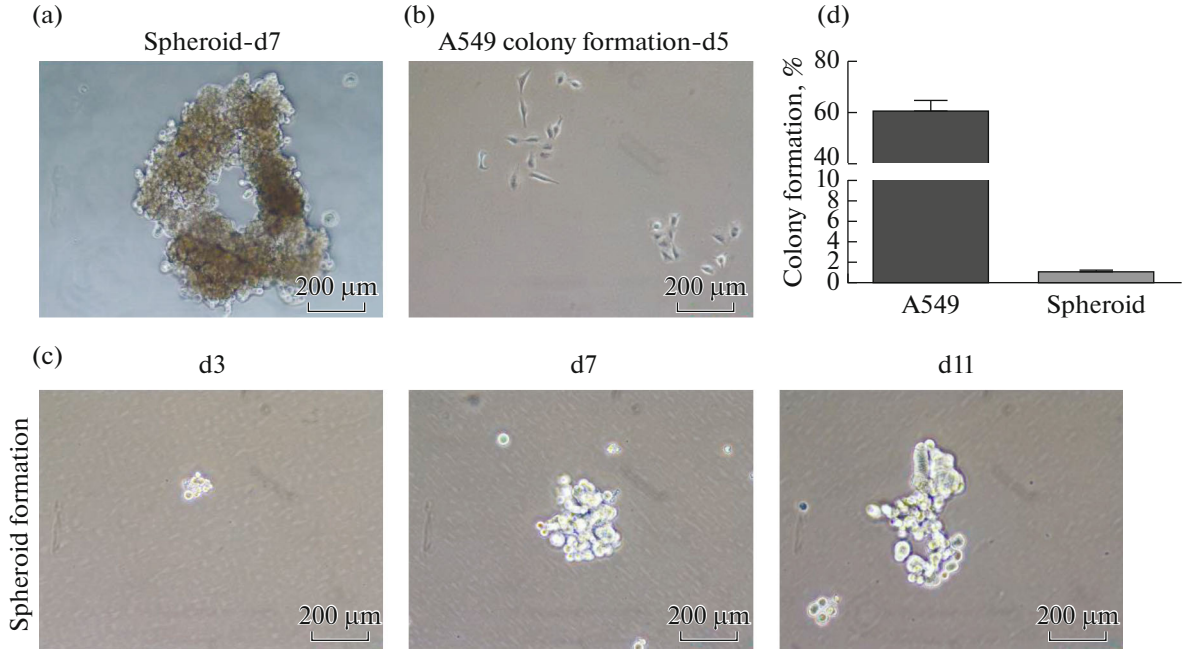

Fig. 1. Representative microscopic images of A549 and spheroid cancer cells with colony/spheroid formation assays. Spheroid cells (a) at day 7 were cultured for (c) spheroid formation assay (days 3, 7, 11). (b) Colony formation of A549 cells at day 5. (d) Colony/spheroid formation efficiencies. Colony and spheroid formation efficiencies were calculated as the percentage of initially seeded cell number (scale bar of a, b: 200 μm; c: 100 μm).

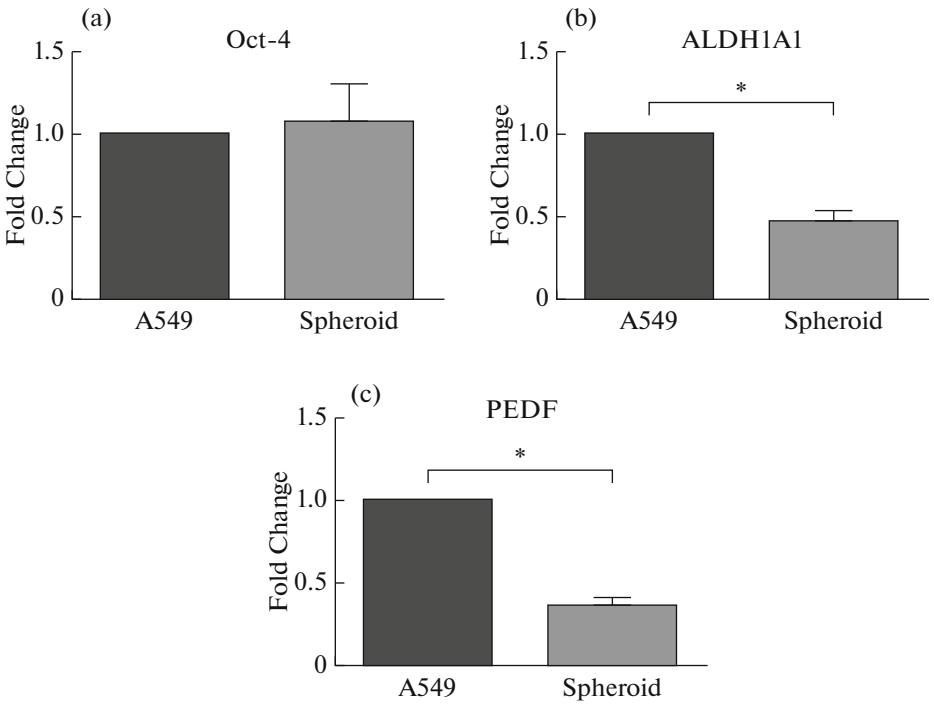

Fig. 2. Gene expression levels in A549 and spheroids analyzed with qRT-PCR. mRNA levels of (a) *Oct4*, (b) *ALDH1A1*, and (c) *PEDF* in A549 parental and cancer spheroid cells. Data were expressed as mean fold change ($2^{-\Delta\Delta Ct}$ method) compared to control (A549). Student's t-test was used for statistical significance (*$p < 0.05$).

mRNA levels decreased significantly compared to parental A549 cells as control ($p < 0.05$, Figs. 2b, 2c). However, there was no significant change in *Oct4* mRNA level in spheroids compared to the control A549 parental cells ($p > 0.05$, Fig. 2a).

DISCUSSION

Reduced ALDH1A1 mRNA Levels with Stable Oct4 in Cancer Spheroids

We analyzed the colony formation efficiencies of A549 and spheroid cancer cells as well as the gene expression levels of prominent stem cell markers *Oct4* and *ALDH1A1* and anti-tumorigenic *PEDF* molecule during transition to spheroids from parental A549 cells. We observed that spheroid formation is executed under serum-free and low-binding culture conditions with ~1% CFE. Similar CFE was also achieved in a previous in vitro study with A549 spheroids under similar conditions (Roudi, 2014). Besides we also found that CFE of A549 parental cells was about 60% consisting of heterogeneous three main types of colonies i.e., paraclone, meraclone and haloclones as described previously (Roudi, 2014).

After that, we performed RT-qPCR analyses of our target genes. We found that *ALDH1A1* mRNA levels significantly reduced during spheroid transformation from adherent A549 cells. As already known from previous studies, CSCs have a distinct expression pattern of a gene cluster providing stemness such as *Oct4, Sox2, CD44, CD133, ALDH1.* Besides, CSCs can also highly express some drug resistance proteins such as ABC transporters (Seo, 2007; Sung, 2008; Kim, 2018). ALDH1 isoenzymes, which catalyze intracellular aldehyde oxidation, were also shown in previous studies to function as drug transporter protein (Sladek, 2003; Kim, 2018). An in vivo study conducted with adenocarcinoma and SCLC lung cancer patients suggested that, immunohistochemical levels of ALDH1A1 and ALDH3A1 isoenzymes increase during early malignant transformation (Patel, 2008). It was also shown in metastatic breast cancer patients that there was a positive correlation between the ALDH1 and multi-drug-resistance proteins (MRPs) in CTCs of the patients (Gradilone, 2011). In a xenograft study conducted with human malignant fibrous histiocytoma cell line, it was shown that $ALDH^+$ subpopulation exhibited higher tumorigenicity and chemoresistance together with elevated expression levels of stemness markers and MRPs (Li, 2015). However, another in vivo study contradicted with these results and claimed that $ALDH^{br}$ (bright) lung cancer cells exhibited similar tumorigenicity with the $ALDH^{lo}$ (dim) but with slower proliferation rate in nude mice (Ucar, 2009). In the same study it was also suggested that although there was no significant difference between the expression patterns of ALDH in primary tumors arisen from $ALDH^{br}$ and $ALDH^{lo}$, $ALDH^{br}$ cells need $ALDH^{lo}$ to be able to sus-

tain tumor mass. There are also contradictory outcomes in the findings of clinical studies. In a study conducted with lung cancer patients it was found that NSCLC patients with ALDH1 expression exhibited a better prognosis compared to ALDH1-negative patients (Dimou, 2012). However, Jiang et al. demonstrated that ALDH1 expression had a positive correlation with NSCLC patients at different disease stages with a lower prognosis and survival (Jiang, 2009). Besides in the same study it was shown that ALDH1$^+$ NSCLC cells, comprising 0.5–3% of total cell population, exhibited stem cell characteristics in vitro. The heterogenic nature of the several other NSCLC cell lines has been previously demonstrated with respect to diverse expression pattern of prominent CSC markers including ALDH1 (Sourisseau, 2014). Based on our findings, the overall reduced expression levels of *ALDH1A1* in lung cancer spheroids can arise from the heterogeneity of the spheroid population in which there are not only CSCs.

As a prominent stem cell marker, Oct4 was shown previously to be highly expressed in drug-enriched A549 CSCs equipped with drug resistance, clone formation, migration, and proliferation (Teng, 2010). Besides, in an in vitro and in vivo study, Oct4 maintained the CSC phenotype of CD133+ positive cells such as self-renewing and chemoresistance (Chen, 2008). But we found that, spheroid formation did not cause an overall increase in *Oct4* mRNA levels. Partially in line with our findings, a previous study performed with tumorspheres cultivated from NSCLC cell lines and patients revealed that *Oct4* mRNA levels did not show a significant change despite increased *ALDH1A1* levels (Herreros-Pomares, 2019). In a spheroid culture model derived from primary human colon cancer, the spheroids overexpressing Oct4 exhibited a similar tumorigenicity and self-renewal potential with their adherent counterpart cancer cells (Qureshi-Baig, 2016). These findings are likely due to the heterogenic nature of cancer cell population regarding the expression of so called a "certain" pattern of stem-cell markers which cannot solely determine their cell fates. In a previous study performed with single-cell-based RT-PCR method it was shown regarding stemness markers of CSCs that, ALDH1$^+$ A549 cells showed an upregulation in gene expression levels of *ALDH1A1* and *ALCAM* whereas there was no significant change in *CD133* or *ABCG2* genes (Xu, 2018). Moreover, in previous clinical studies, the isolated CSCs from the patients with same cancer type exhibited diverse expression patterns of prominent CSC markers (Singh, 2015). Taken together the previous findings, it can be inferred that spheroids, as a heterogeneous cell population, can exhibit a highly versatile gene expression profile at single cell level.

Reduction of PEDF as a Prevention of Potent "Threat" by Spheroids

PEDF acts as an anti-tumorigenic and anti-metastatic protein by enhancing cellular differentiation and inducing apoptosis but its role has not been shown in A549 cancer spheroid cells yet (Belkacemi, 2016; Zhou, 2016; Ribaux, 2019). Regarding the gene expression levels of PEDF in spheroids, we showed for the first time that spheroid transformation of parental A549 cells caused reduction in *PEDF* mRNA levels. It was demonstrated in a mouse model of melanoma that the knockout of *PEDF* transformed the cancer cells into a more metastatic state (Nwani, 2016). In clinical studies, the downregulation of PEDF has been reported to be associated with poor prognosis and higher metastatic rate in several cancer types including lung cancer (Zhang, 2006; Chen, 2009). Furthermore, the increased levels of PEDF were asserted to be correlated with a better prognosis in lung cancer patients (Zhou, 2016). In this regard, the reduced expression levels of *PEDF* in the present study can be inferred as an intrinsic suppression mechanism in spheroids to gain a more metastatic phenotype, to inhibit cellular differentiation, and to reach a higher tumorigenicity.

CONCLUSIONS

Although "stemness" of CSCs is characterized by elevated stem cell markers e.g., Oct4 and ALDH1, spheroid cells are a mixture of different cell subsets in which only SP has these specific stem cell characteristics. As to our knowledge, we for the first time assessed the altered gene expression levels of *PEDF* and prominent cancer stem cell markers during spheroid transition of adherent lung cancer cells. Since we did not enrich or isolate these specific CSCs, there was no overall increase in either of stemness markers. In contrary, we found a significant decrease in *ALDH1A1*. Reduced PEDF levels, however, can be a defense mechanism of spheroid cells against anti-tumorigenic effects of PEDF. For a better understanding, effect of *PEDF* on spheroid cells should be evaluated at both gene and protein levels.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflicts of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

AUTHOR CONTRIBUTION

MYT designed the study. MYT and HMO performed the experiments. M.Y.T., H.M.O., G.G.D., and M.U.K. analyzed the data statistically and M.Y.T. prepared all figures. M.Y.T. prepared tables. All authors wrote/drafted/edited/revised the manuscript and interpreted the results. All authors read, edited, and gave an approval for the final version of the present manuscript. All authors are aware of the order of authorship and that no further changes in authorship will be performed after submission.

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