Novel markers of human ovarian granulosa cell differentiation toward osteoblast lineage: A microarray approach

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Abstract. Under physiological conditions, human ovarian granulosa cells (GCs), are responsible for a number of processes associated with folliculogenesis and oogenesis. The primary functions of GCs in the individual phases of follicle growth are: Hormone production in response to follicle stimulating hormone (FSH), induction of ovarian follicle atresia through specific molecular markers and production of nexus cellular connections for communication with the oocyte. In recent years, interest in obtaining stem cells from particular tissues, including the ovary, has increased. Special attention has been paid to the novel properties of GCs during long-term *in vitro* culture. It has been demonstrated that the usually recycled material in the form of follicular fluid can be a source of cells with stem-like properties. The study group consisted of patients enrolled in the *in vitro* fertilization procedure. Total RNA was isolated from GCs at 4 time points (after 1, 7, 15 and 30 days of culture) and was used for microarray expression analysis (Affymetrix® Human HgU 219 Array). The expression of 22,480 transcripts was examined. The selection of significantly altered genes was based on a P-value <0.05 and expression higher than two-fold. The leucine rich repeat containing 17, collagen type I α1 chain, bone morphogenetic protein 4, twist family bHLH transcription factor 1, insulin like growth factor binding protein 5, GLI family zinc finger 2 and collagen triple helix repeat containing genes exhibited the highest changes in expression. Reverse-transcription-quantitative PCR was performed to validate the results obtained in the analysis of expression microarrays. The direction of expression changes was validated in the majority of cases. The presented results indicated that GCs have the potential of cells that can differentiate towards osteoblasts in long-term *in vitro* culture conditions. Increased expression of genes associated with the osteogenesis process suggests a potential for uninduced change of GC properties towards the osteoblast phenotype. The present study, therefore, suggests that GCs may become an excellent starting material in obtaining stable osteoblast cultures. GCs differentiated towards osteoblasts may be used in regenerative and reconstructive medicine in the future.

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Introduction

A mature ovarian follicle consists of several basic elements that together form one functional unit. Theca externa is located the most externally, followed by theca interna, while granulosa cells (GCs) and oocytes are located inside the ovarian follicle. Several types of follicular GCs are distinguished in the ovarian follicle: Cells lining the inner part of the ovarian follicle, adjacent to the basal lamina are called mural GCs, followed by the layer forming the cumulus oophorus and cells directly surrounding the oocyte-corona radiata. The basic functions of GCs in the individual phases of follicle growth are the production of hormones in response to follicle stimulating hormone (FSH), induction of ovarian follicle atresia through specific molecular markers, as well as the production of nexus cellular connections for communication with the oocyte (1-5). The well-known physiological properties of GCs have influenced the intensive development of fields related to assisted reproduction techniques in humans and animals (6-9).

In recent years, an increase of interest and intense development of domains related to stem cells has been observed. The term 'stem cell' describes cells that have the ability to self-renew and differentiate into more targeted cell types.

There is a hierarchy of stem cells from those that give rise to all types of cells to those that only differentiate into tissue and organ-specific products. We, therefore, distinguish: Totipotent stem cells-giving rise to embryo cells and extraembryonic tissue; pluripotent stem cells-giving rise to all three germ layers; multipotent stem cells-giving rise to cells from two or one germ layer and unipotent stem cells-tissue-directed, giving rise only to specific cell lines (10). The research suggests that the stem cell reservoir is located in every mature tissue, because cells undergoing apoptosis are continuously replaced by new cells. It is suggested that the biggest stem cell pools are located in the liver, lungs and pancreas (11-15). In terms of clinical approaches, one of the most promising types of such cells are mesenchymal stem cells (MSCs). MSCs are multipotent progenitor cells that have the ability to differentiate towards bone, cartilage and adipose cells. These cells are obtained primarily from fetal tissues (placenta, umbilical cord blood, umbilical cord), also being found in the body of an adult organism (16-19). The main source of adult MSCs in recent years has been the bone marrow and adipose tissue (20). Recent studies indicate that the ovary can also be a source of stem cells. Kossowska-Tomaszczuk *et al* (21) indicates surprising stem-like properties of GCs. According to her research, GCs *in vitro* have the properties similar to those of MSCs. Other authors also point out that GCs have stem cell properties, but not as broad as those of MSCs and pluripotent stem cells (22,23). The presented research suggests that GCs, routinely disposed of during the *in vitro* fertilization procedure, may become a valuable source of cells used to obtain osteoblast populations. Such osteoblasts could be used in the treatment of diseases related to skeletal system pathologies.

Materials and methods

Part of the material and methods section is based on other publications of the same research team, presenting results from the same cycle of studies related to human ovarian GCs (24,25).

Granulosa cell collection. The study group consisted of 8 patients, aged 18-40 years, enrolled in *in vitro* fertilization (IVF) procedure in the Division of Infertility and Reproductive Endocrinology, Poznan University of Medical Sciences, Poland. Follicular fluid containing the GCs from patients undergoing *in vitro* fertilization (IVF) procedures was collected.

The IVF procedure was based on a controlled ovarian hyperstimulation protocol adapted to the patient's initial cause of infertility, as well as predicted and current ovarian response. Stimulation with human recombinant FSH (Gonal-F; Merck-Serono; Merck KGaA) and highly purified human menopausal gonadotropin (hMG-HP; Menopur; Ferring) has been performed according to protocol. Gonadotropin-releasing hormone (GnRH) antagonist-cetrorelix acetate (Cetrotide; Merck-Serono; Merck KGaA), injection has been given at the appropriate dose to suppress the function of the pituitary gland. Induction of ovulation was based on the subcutaneous injection of 6.500 h of human chorionic gonadotropin (hCG; Ovitrelle; Merck-Serono; Merck KGaA). The doses of gonadotropins and GnRH antagonist have been precisely controlled and recorded for every patient. The follicular fluid has been collected during transvaginal ultrasound-guided oocyte pick-up, 36 h after administration of human chorionic gonadotropin. GCs have been taken from follicles with a diameter of over 16 mm. Directly after ovarian puncture, the complete content of the ovarian follicle (follicular fluid containing GCs and oocytes) was passed on to a qualified embryologist that extracted all of contained oocytes that were subsequently used in further stages of the IVF procedure (conducted at Division of Infertility and Reproductive Endocrinology, Department of Gynecology, Obstetrics and Gynecological Oncology, Poznan University of Medical Sciences, Poznan, Poland). Meanwhile, the remaining granulosa cell containing follicular fluid, usually discarded after this step, was passed on to the employees of Department of Anatomy, Poznan University of Medical Sciences, in which the further research was conducted. Patients with a potential risk of inadequate ovarian stimulation-according to Bologna criteria of poor ovarian responders, published by European Society of Human Reproduction and Embryology (ESHRE) in 2011 (26) have been excluded, accepting serum antimullerian hormone (AMH) 0,7 ng/ml as a cut-off value. Moreover, patients with serum level of FSH above 15 mU/ml on the 2nd-3rd day of the cycle, as well as patients with polycystic ovary syndrome and endometriosis, have also been excluded from the study. Only ovarian GCs, usually a part of the discarded remnant material of the IVF procedure, were used in the research. This study has been approved with resolution 558/17 by Poznan University of Medical Sciences Bioethical Committee. All participants gave their written informed consent for use of their material in research.

Primary cell culture. The GCs, suspended in follicular fluid, were washed twice by centrifugation at 200 x g for 10 min at RT. Medium consisted of Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich Co.; Merck KGaA), 2% fetal bovine serum FBS (FBS; Sigma-Aldrich; Merck KGaA), 4 mM L-glutamine (Invitrogen; Thermo Fisher Scientific, Inc.), 10 mg/ml gentamycin (Invitrogen; Thermo Fisher Scientific, Inc.), 10,000 U/ml penicillin, and 10,000 μg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were cultivated at 37˚C under aerobic conditions (5% CO_2) in 25 cm^3 culture flasks (Corning Inc., Corning, NY, USA). The cells were passaged upon reaching 90% confluence; they were detached with 0.05% trypsin-EDTA (Invitrogen; Thermo Fisher Scientific, Inc.) for 1-2 min and counted using an ADAM Cell Counter and Viability Analyzer (Bulldog Bio). After counting, the cells were seeded onto a number of flasks appropriate for total cell number (2-3x10^6 cells per 25 cm^3 flask). GCs were then cultivated for 30 days, the morphology was checked daily and photographed using an Olympus inverted microscope (Olympus). The medium was changed twice a week. Finally, total RNA was isolated from GCs after 1, 7, 15 and 30 days. The viability of each collected sample was tested using the ADAM CCVA, with only samples containing 95% or more viable cells used for subsequent molecular analyses.

Total RNA isolation. RNA was isolated at 4 time periods, after 1, 7, 15, and 30 days cultivation. The Chomczyński-Sacchi method was used to isolate the total RNA (27). The GCs were suspended in 1 ml mixture of guanidine thiocyanate and

phenol in monophase solution (TRI Reagent®; Sigma-Aldrich; Merck KGaA). In the next step, the chloroform was added and centrifuged to separate 3 phases. RNA has been located in an aqueous phase. The resulting RNA was intact with no contaminating DNA and protein. In the last step, the RNA has been precipitated with 2-propanol (Sigma-Aldrich, cat. no. I9516), in amount accurate per 1 ml of TRI-reagent and has been washed with 75% ethanol. Resulting RNA has been used for further analysis. The total mRNA was determined from the optical density at 260 nm and the RNA purity was estimated using the 260/280 nm absorption ratio (NanoDrop spectrophotometer, Thermo Scientific). Samples with absorbance ratio 260/280 greater than 1.8 have been used to the presented study.

Microarray expression analysis. The microarray procedure was conducted according to protocols used in previous studies of our team (5,28-32). Total RNA (100 ng) from each pooled sample was subjected to two rounds of sense cDNA amplification (Ambion® WT Expression Kit). The obtained cDNA was used for biotin labelling and fragmentation using Affymetrix GeneChip® WT Terminal Labeling and Hybridization (Affymetrix). Biotin-labelled fragments of cDNA (5.5 μg) were hybridized to the Affymetrix® Human Genome U219 Array (48˚C /20 h). Microarrays were then washed and stained according to the technical protocol using the Affymetrix GeneAtlas Fluidics Station. The array strips were scanned employing Imaging Station of the GeneAtlas System. Preliminary analysis of the scanned chips was performed using Affymetrix GeneAtlas™ Operating Software. The quality of gene expression data was confirmed according to the quality control criteria provided by the software. The obtained CEL files were imported into downstream data analysis software.

Reverse transcription-quantitative PCR (RT-qPCR). The RT-qPCR method was performed to confirm the results obtained in the analysis of expression microarrays. Three genes were selected from each heatmap: The ones showing highest, lowest, and most intermediate-level of expression. Changes in the level of expression of those genes were then examined. In each group, three independent samples were analyzed, each coming from a different patient (referred to as a biological repeat). Each test was performed in 3 replicates. Reverse transcription was based on the protocols and reagents of SABiosciences (RT^2 First Stand Kit-330401), using a Veritimer 96 well Thermal Cycler. 1 μg of each gene's RNA transcript was used for reverse transcription. qPCR was performed using the Light Cycler® 96 (Roche Diagnostic GmbH, Germany), RT^2 SYBR® Green ROX™ qPCR Master Mix (Qiagen Sciences, Inc.,) and sequence-specific primers (Table I). *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin (ACTB)* and *hypoxanthine phosphoribosyltransferase 1 (HRPT1)* were used as reference genes. Gene expression was analyzed using the $2^{-\Delta\Delta Cq}$ method (33). The qPCR starters were designed using the Primer3Plus software (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi).

Statistical analysis. All of the presented analyses and graphs were performed using Bioconductor and R programming languages. Each CEL file was merged with a description file. In order to correct background, normalize, and summarize results, we used the Robust Multiarray Averaging (RMA) algorithm. To determine the statistical significance of the analyzed genes, moderated t-statistics from the empirical Bayes method were performed. The obtained P-value was corrected for multiple comparisons using Benjamini and Hochberg's false discovery rate. The selection of significantly altered genes was based on a P-value beneath 0.05 and expression higher than two-fold. The differentially expressed gene list (separated for up- and down-regulated genes) was uploaded to the DAVID software (Database for Annotation, Visualization and Integrated Discovery) (34).

Subsequently, sets of differentially expressed genes from selected GO BP terms were applied to STRING software (Search Tool for the Retrieval of Interacting Genes/Proteins) for interaction prediction. STRING is a huge database containing information about protein/gene interactions, including experimental data, computational prediction methods and public text collections.

Finally, the functional interactions between genes that belong to the chosen GO BP terms were investigated by REACTOME FIViz application to the Cytoscape 3.6.0 software. The ReactomeFIViz app is designed to find pathways and network patterns related to cancer and other types of diseases. This app accesses the pathways stored in the Reactome database, allowing to do pathway enrichment analysis for a set of genes, visualize hit pathways using manually laid-out pathway diagrams directly in Cytoscape, and investigate functional relationships among genes in hit pathways. The app can also access the Reactome Functional Interaction (FI) network, a highly reliable, manually curated pathway-based protein functional interaction network covering over 60% of human proteins.

Additionally, a statistical analysis of the RT-qPCR results (dependent sample Student's t-test corrected for multiple comparisons using Benjamini and Hochberg's false discovery rate) was conducted for every analyzed sample mean. Samples were only considered further if $P<0.05$. This analysis employed the Real Statistics Resource Pack add-on for MS Excel 2016 (Microsoft Corporation).

Results

Whole transcriptome profiling by Affymetrix microarray allowed us to analyze the expression changes in GCs, after 1, 7, 15 and 30 days of culture. By Affymetrix® Human HgU 219 Array, we examined the expression of 22480 transcripts. Genes with a fold change higher then abs (2) and with a corrected P-value lower than 0.05 were considered as differentially expressed. This set of genes consisted of 2278 different transcripts.

DAVID (Database for Annotation, Visualization and Integrated Discovery) software was used for extraction of the gene ontology biological process terms (GO BP). Up and down-regulated gene sets were subjected to the DAVID search separately and only gene sets of adj. P-values <0.05 were selected. The DAVID software analysis showed that differentially expressed genes belonged to 582 Gene Ontology Biological Process (GO BP) terms and 45 KEGG pathways. In this report, we focused on 'osteoblast differentiation' GO BP term. This set of genes was subjected to hierarchical

Table I. Oligonucleotide sequences of primers used for RT-qPCR analysis.

Gene name	Primer sequence (5'-3')	Product size (bp)
SYNCRIP	F: TGTGGGAAAGATCCCAAGAG	231
	R: TTGGCAACTGAGATGCAGAC	
MRC2	F: CACCAAACTCCGGTATTGCT	189
	R: TGGATCTCGGGTTCTGATTC	
RRAS2	F: AGCACGGCAGCTTAAGGTAA	165
	R: TGGCAGCCTTTCTTGTCTTT	
TPM4	F: TTGAGGAGGAGTTGGACAGG	159
	R: GCTGCATCTCCTGAATCTCC	
SPP1	F: GCCGAGGTGATAGTGTGGTT	242
	R: GTGGGTTTCAGCACTCTGGT	
FHL2	F: CTCATCCAAGTGCCAGGAAT	175
	R: CTCATAGCAGGGCACACAGA	
SKI	F: CAGCAGAAGGTTGTGAGCAG	165
	R: CGAGTCCTTGTCCTCCTCTG	
EPHA2	F: GAGGGCGTCATCTCCAAATA	236
	R: TCAGACACCTTGCAGACCAG	
CYR61	F: CTCCCTGTTTTTGGAATGGA	241
	R: TGGTCTTGCTGCATTTCTTG	
CDK6	F: TGCACAGTGTCACGAACAGA	150
	R: ACCTCGGAGAAGCTGAAACA	
SFRP1	F: CGAGTTTGCACTGAGGATGA	190
	R: GAAGTGGTGGCTGAGGTTGT	
CTHRC1	F: GCTCACTTCGGCTAAAATGC	165
	R: CCACAGAAGAAGTGCGATGA	
CREB3L1	F: AGGTGGAGACCCTGGAGAAT	223
	R: AGGGGGTCTTCCTTCACAGT	
GLI2	F: CACCAACCAGAACAAGCAGA	246
	R: ACCTCAGCCTCCTGCTTACA	
IGFBP5	F: GAGCTGAAGGCTGAAGCAGT	237
	R: GAATCCTTTGCGGTCACAAT	
TWIST	F: GTCCGCAGTCTTACGAGGAG	159
	R: CCAGCTTGAGGGTCTGAATC	
BMP4	F: CTGGTCCACCACAATGTGAC	162
	R: CGATCGGCTAATCCTGACAT	
COL1A1	F: GTGCTAAAGGTGCCAATGGT	228
	R: CTCCTCGCTTTCCTTCCTCT	
LRRC17	F: CAACCCCTGGCACTGTACTT	225
	R: ACCTCAGGCTTGATGACTGG	
GAPDH	F: TCAGCCGCATCTTCTTTTGC	90
	R: ACGACCAAATCCGTTGACTC	
ACTB	F: AAAGACCTGTACGCCAACAC	132
	R: CTCAGGAGGAGCAATGATCTTG	
HPRT	F: TGGCGTCGTGATTAGTGATG	141
	R: ACATCTCGAGCAAGACGTTC	

F, forward; R, reverse. RT-qPCR, reverse transcription-quantitative PCR.

clusterization procedure and presented as a heatmap (Fig. 1). The gene symbols, fold changes in expression, Entrez gene IDs and corrected P-values of these genes were shown in Table II.

STRING interaction network was generated among differentially expressed genes belonging to each of selected GO BP terms. Using such a prediction method provided

Table II. Gene symbols, fold changes in expression, Entrez gene IDs and corrected P-values of studied genes.

Symbol	Entrez Gene ID	Fold change D7/D1	Fold change D15/D1	Fold change D30/D1	Adj. P-value D7/D1	Adj. P-value D15/D1	Adj. P-value D30/D1
SYNCRIP	10492	2.060	1.867	1.885	0.013	0.018	0.016
MRC2	9902	2.337	2.031	1.974	0.018	0.028	0.028
RRAS2	22800	2.242	2.358	2.427	0.025	0.019	0.015
TPM4	7171	2.538	2.478	2.493	0.013	0.012	0.011
SPP1	6696	9.788	0.953	2.840	0.028	0.965	0.188
FHL2	2274	3.073	3.898	2.849	0.028	0.014	0.028
SKI	6497	2.463	2.503	3.342	0.003	0.002	0.001
EPHA2	1969	4.820	2.854	3.519	0.003	0.008	0.004
CYR61	3491	4.318	4.249	4.154	0.001	0.001	0.001
CDK6	1021	3.725	3.762	4.232	0.024	0.022	0.014
SFRP1	6422	5.257	6.419	5.765	0.009	0.006	0.006
CTHRC1	115908	3.349	4.023	6.926	0.040	0.023	0.007
CREB3L1	90993	6.916	5.727	12.541	0.004	0.005	0.001
GLI2	2736	6.935	7.104	14.113	0.019	0.016	0.006
IGFBP5	3488	12.733	16.566	17.230	0.014	0.009	0.008
TWIST1	7291	13.411	16.061	20.741	0.001	0.001	0.001
BMP4	652	10.489	17.221	21.243	0.020	0.010	0.007
COL1A1	1277	8.195	19.443	85.847	0.028	0.009	0.002
LRRC17	10234	3.985	34.428	103.392	0.016	0.001	0.000

D, day of *in vitro* culture.

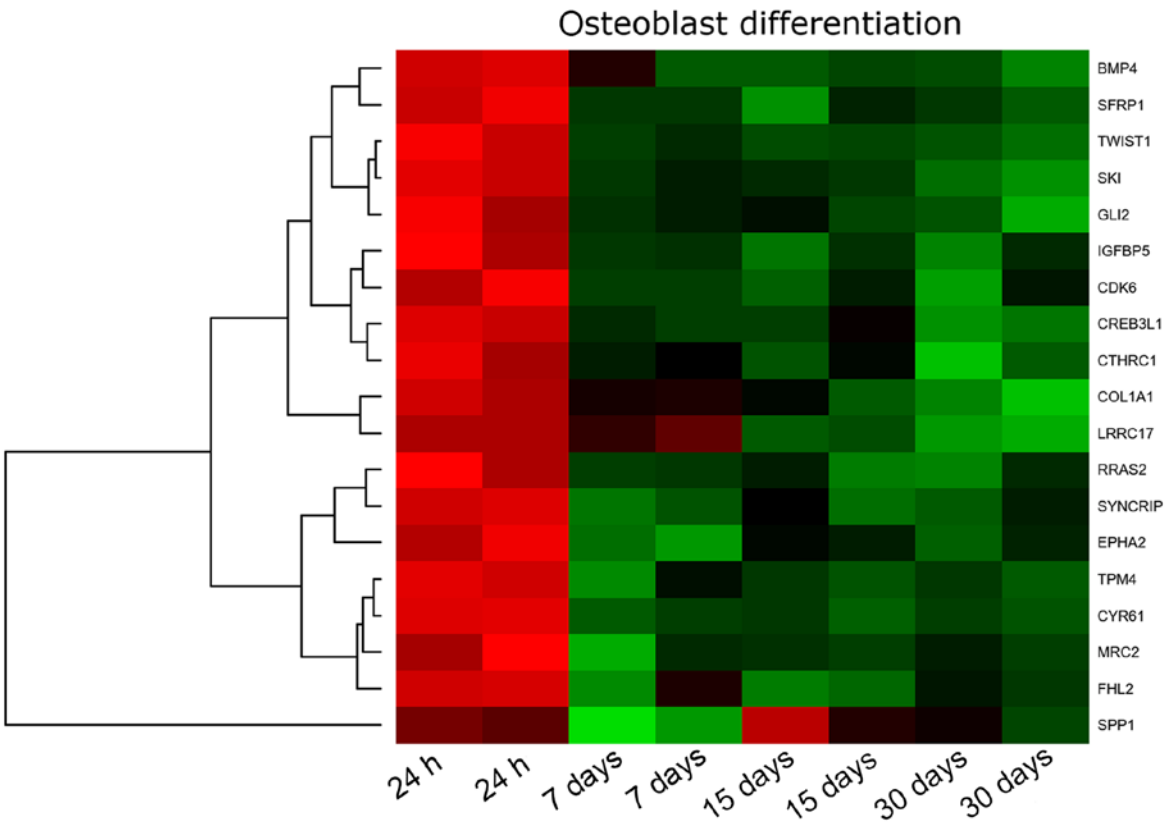

Figure 1. Heat map representation of differentially expressed genes belonging to the 'osteoblast differentiation' Gene Ontology biological process term. Arbitrary signal intensity acquired from microarray analysis is represented by colors. Green represents higher expression, and represents red lower expression. Log2 signal intensity values for any single gene were resized to Row Z-Score scale (-2, lowest expression; +2, highest expression for the single gene). D, days of *in vitro* culture; H, hours of *in vitro* culture.

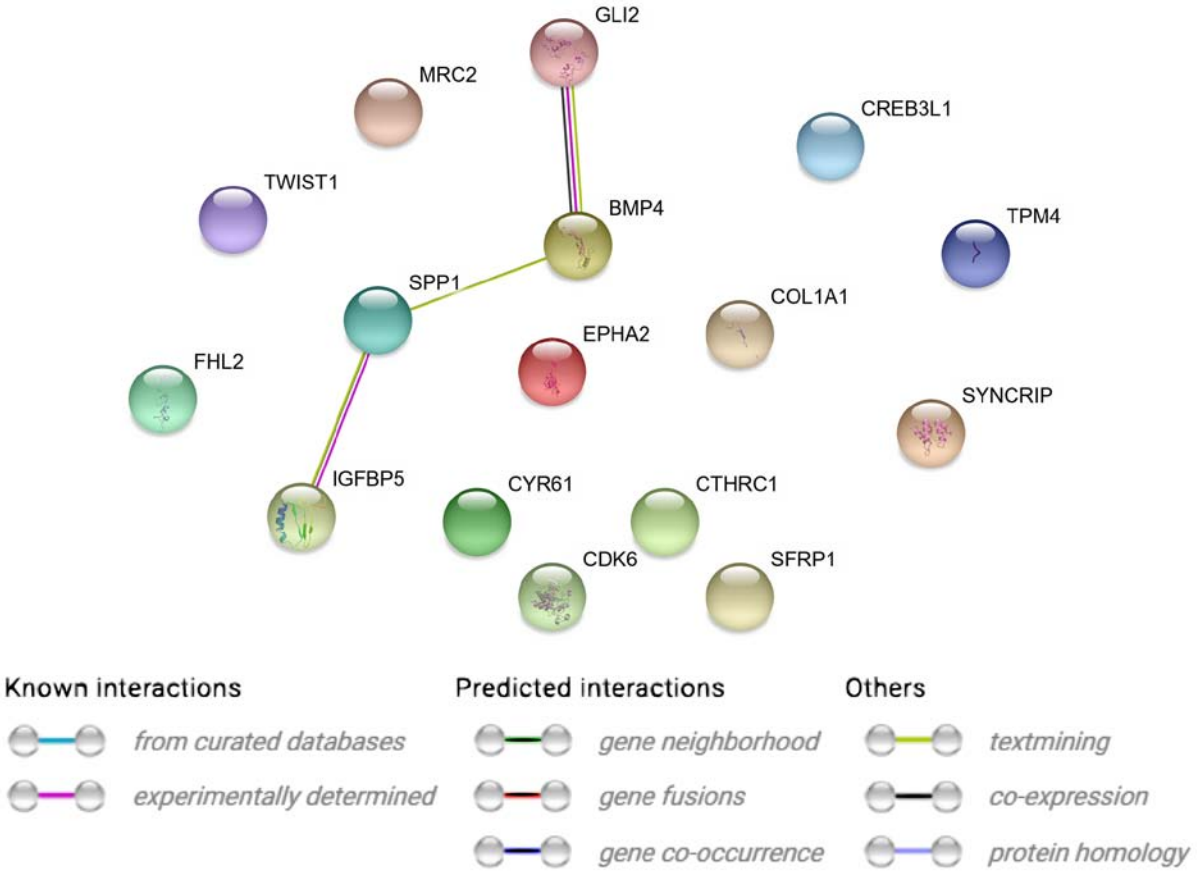

Figure 2. STRING-generated interaction network of differentially expressed genes belonging to the 'osteoblast differentiation' Gene Ontology biological process terms. The intensity of the edges reflects the strength of the interaction score.

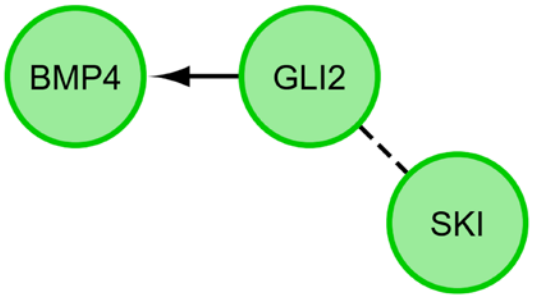

Figure 3. FI between differentially expressed genes belonging to the 'osteoblast differentiation' Gene Ontology biological process terms. '->' represents activating/catalyzing and '---' predicted FIs. BMP4, bone morphogenetic protein 4; FI, functional interaction; GLI2, GLI family zinc finger 2; SKI, SKI proto-oncogene.

us with a molecular interaction network formed between protein products of studied genes (Fig. 2). Finally, we investigated the functional interactions between chosen genes with REACTOME FIViz app to Cytoscape 3.6.0 software. The results were shown in Fig. 3.

RT-qPCR was conducted to validate the results obtained during microarray analysis. The outcomes were presented and compared in a form of a bar graph (Fig. 4).

As can be seen, the direction of changes in expression was confirmed in most examples. Nevertheless, the microarray approach, used to analyze the full transcriptome of the cells, is largely qualitative, which can be observed as validation of the results with quantitative RT-qPCR in two examples gives variable results. For SPP1 transcript expression level, microarray results indicate downregulation in 15 days of cell culture, while RT-qPCR results give a different direction of changes. A similar situation may be observed for RRAS1 mRNA levels. This might be due to the fact that the microarrays account for multiple available exons forming many variants of the expressed gene, which is not usually the case with RT-qPCR, as it probes for a specific gene sequence. Likewise, the scale of differences in transcript levels varied between both of the methods analysed.

Discussion

The process of MSC differentiation towards osteoblasts is regulated by a number of transcription factors, the list of which is still incomplete and systematically supplemented and updated (35,36). During the development of the skeleton, MSCs may give rise to two cell lines: i) Osteoblasts and ii) chondroblasts. The dual development potential of MSC is made possible due to expression of two basic bone formation regulators: Runx-2 (CBFA-core binding factor Ralpha/osteoblast-specific factor 2-OSF-2) and SOX-9. The further way of differentiation (towards osteoblasts or chondroblasts) is decided by the inclusion of further transcription factors. Osterix (OSX), and Runx-2 are some of the factors that facilitate the osteogenesis process (37,38). The increase in expression of OSX factor influences the increase of expression

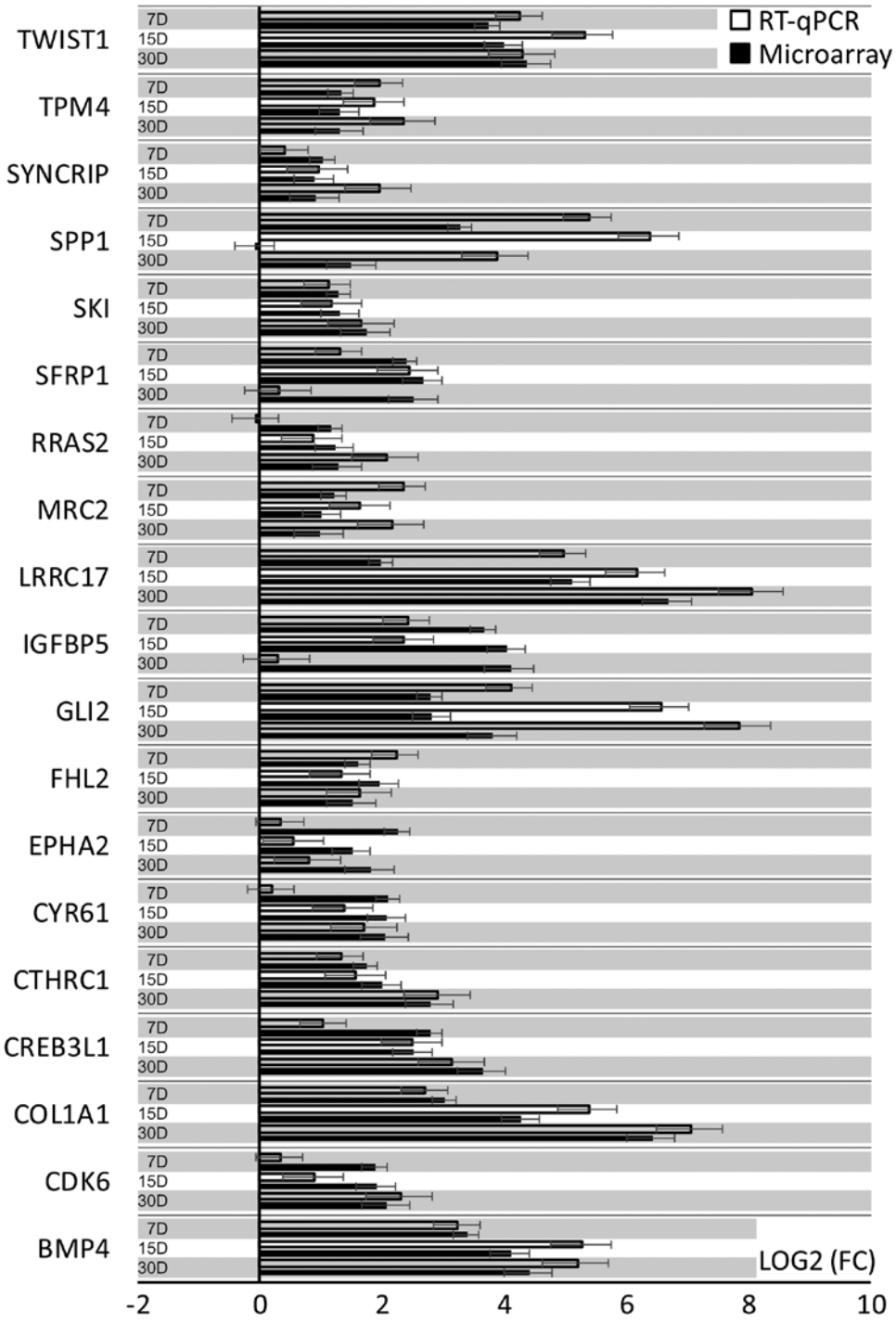

Figure 4. Results of the RT-qPCR validation of microarray results. Presented as a bar graph. FC, was presented in its logarithmic form [Log(FC)] to provide clear comparability of the results. The error bars represent the SEM. All presented sample means were deemed to be statistically significant ($P<0.05$). D, days of *in vitro* culture; FC, fold change; RT-qPCR, reverse transcription-quantitative PCR.

and secretion of osteoblast-specific proteins-collagen type I, osteopontin, sialoprotein and alkaline phosphatase (39). Other important factors regulating osteoblastogenesis are involved in the Wnt/β-catenin signalling pathway. Activation of Wnt pathway proteins stimulates osteoblast proliferation (36,38). Bone morphogenetic proteins (BMPs) are yet another important group of factors regulating the process of osteoblastogenesis. These proteins have a stimulating effect on osteoblast activity through BMP serine-threonine kinase receptors. Many other factors also contribute to the stimulation of osteoblast activity such as parathormone (PTH)-acting via insulin-like growth factor (IGF), dexamethasone, lectin etc. (40-43).

As mentioned above, the literature provides a number of factors that influence the differentiation of MSCs towards osteoblasts *in vitro*. Many authors indicate that the source of these stem cells is also the ovary. Kossowska-Tomaszczuk *et al* (21) were the first to suggest that GCs have the potential of stem cells (mesenchymal stem cells), due to the expression of markers characteristic for this type of cells. It was the first to prove that GCs can differentiate into osteoblasts in long-term

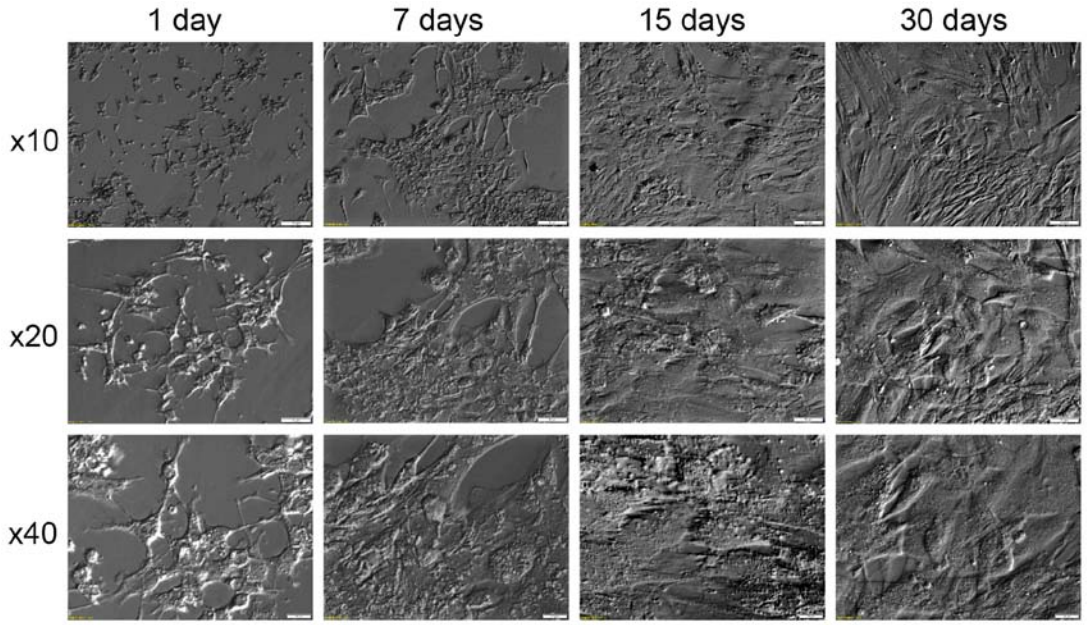

Figure 5. Morphology of human ovarian GCs in long-term *in vitro* culture. The magnifications are indicated on the left in the figure. Magnification, x10, x20 and x40. GCs, granulosa cells.

in vitro culture under the influence of a suitable differentiating medium. GCs thus shed new properties and could be successfully used as a starting material for obtaining stable populations of osteoblasts used in regenerative medicine of skeletal-related disorders (21,44,45). The results of the presented studies confirm the possibility of differentiating GCs towards osteoblasts. We can observe that GCs is subject to such differentiation without differentiating factors constituting the supplement of the culture medium. We can, therefore, conclude that GCs undergoes a number of changes in gene expression during long-term *in vitro* culture, the effect of which is their entry into the pathway of osteoblast differentiation.

The presented studies also indicate that GCs may differentiate towards osteoblasts under long-term *in vitro* culture conditions. Moreover, it was shown that GCs express genes characteristic for this process, which can be considered genetic markers of differentiation of GCs towards osteoblasts. GCs in the presented studies were cultivated without the addition of supplements considered necessary for the process of cell differentiation towards osteoblasts. The basal medium did not contain any supplements such as dexamethasone, BMP-2, vitamin D3, ascorbic acid, β-glycerophosphate, valproic acid, which are considered to be key factors osteoblast differentiation (15,19,46). The results of the presented research on the potential of GCs differentiation towards osteoblasts are confirmed in the literature of recent years (21,23,44).

The 'osteoblast differentiation' ontological group defines a group of genes responsible for the biological process during which the differentiation of less specialized cells towards osteoblasts occurs. The included heat map presents a set of genes that are characteristic of the process of GCs differentiation towards osteoblasts.

As can be seen in the attached table, during the 30-day *in vitro* culture, the expression of 19 genes is changed, with *SYNCRIP, MRC2, RRAS2* and *TPM4* exhibiting the lowest expression, and *LRRC17, COL1A1, BMP4, TWIST1, IGFBP5, GLI2, CTHRC1* showing the highest expression. This report is focused on the genes of the highest expression and their mutual relation.

Analyzing the relationships and interactions between the 19 genes of interest, we can only observe some between two pairs of genes (*BMP4* and *GLI2* and *SPP1* and *IGFBP5*).

The highest expression from all genes has been demonstrated by *LRRC17* (*Leucine-rich repeat containing 17)*. It is not only one of the genes that regulate the osteoblastogenesis process, but also a gene that is expressed by the ovary. The above result, on one hand, can confirm that the obtained cells are cells derived from the ovary, but on the other hand can be a factor regulating the process of differentiation of GCs towards osteoblasts (47,48). It is a gene that is highly expressed in osteoblasts under physiological conditions (47). The high expression change of this gene during long-term *in vitro* GC culture indicates that these cells can spontaneously gain osteoblast properties.

Another very important gene demonstrating the differentiation of a given population towards osteoblasts is the expression of the *collagen type I* gene and protein. The expression of collagen type I is significantly increased after 30 days of GC *in vitro* culture. We can, therefore, suppose that the process of GCs differentiation towards osteoblasts is also regulated by typical factors of MSC osteoblastogenesis. *COL1A1 (Collagen type I alpha 1 chain)* is a gene providing inductions for the synthesis of a large molecular molecule called collagen type I. Collagens are a family of proteins that are part of most organs in the human body: They build cartilage, tendons, skin, sclera, and above all bones. Collagen molecules form long fibrils, connected by transverse bonds between them in intercellular spaces. Such structure and interactions between collagen fibres are called cross-linking, which results in the formation of very strong type 1 collagen fibres (49). The expression of the *COL1A1* gene indicates that GCs have the potential to differentiate towards the bone tissue. Under physiological conditions,

the *COL1A1* gene is not expressed in GCs. Only the presence of collagen type I in the theca cells of the ovarian follicle has been proven (50,51). However, these are not the subject of the research presented. Only GCs building an internal layer of ovarian follicle were used in the study.

Osteogenesis is the process leading to the formation of bone tissue. It begins during the formation of the embryo and continues throughout the entire life of the organism by maintaining a balance between bone formation and resorption (52). Bone morphogenetic proteins belong to the superfamily of TGF-βs (*Transforming growth factors* β). The family of these proteins is responsible for the formation of bone and cartilage *in vivo* but also fulfils important roles in the female reproductive system (53,54). One of the strongest inductors in bone formation through osteoblast differentiation stimulation is BMP-4. Under physiological conditions, BMP-4 transduced signals through heterodimer formation of Type II and Type I cognate complex (BMPRIA and BMPRIB) through serine/threonine receptors. This leads to phosphorylation of Sma and Mad proteins playing an important role in the differentiation of cells derived from the mesenchymal line (55-57). The group of BMP proteins also plays a role in the regulation of follicular development and has an effect on GCs proliferation and steroidogenesis. Tanwar and Mcfarlane (58) indicate, in their mice studies, that BMP-4 is expressed in the ovary, uterus and oviduct epithelium. It has also been shown that BMP-4 acts as a paracrine/autocrine modulator of steroidogenesis of GCs cells. BMP-4 together with BMP-6, BMP-7 stimulates Smad-1 accumulation and release of estradiol (E2) stimulated by IGF (59,60). Other studies have shown that another types of BMP, above all BMP-15, are necessary for the proper functioning of the reproductive system of the female. It is BMP-15 that affects the proliferation and differentiation of GCs into individual layers within the follicle (61). The presented studies do not clearly indicate whether the expression of BMP-4 is associated with the the process of steroidogenesis occurring *in vitro* culture or whether this expression is associated with osteogenesis. In a broad sense, we can conclude that the presence of other genes involved in the BMP-4 osteogenesis process could confirm its occurrence in the culture. This conclusion is supported by the expression of the previously mentioned SPP1 gene (*secreted phosphoprotein; osteopontin*) (62). SPP1 is a gene responsible for the proper bone mineralization in the process of osteogenesis. Kim *et al* (63) suggested that SPP1 could be a marker of ovarian cancer. Under physiological conditions, the expression of SPP1 increases in the antral follicles (64) suggesting that SPP1 does not indicate a differentiation of GCs towards osteoblasts. Kulterer *et al* (65) proved that during the differentiation of MSCs towards osteoblasts, SPP1 and COL1A1 are expressed (66). Our research confirms this scheme; however, it should be emphasized that in the presented results P-values were <0.05 only after 7 days of culture, bringing their actual significance into question. In addition, another gene supporting the GCs' tendency for osteogenic differentiation in long-term *in vitro* is the GLI2 (*GLI-Kruppel Family Member 2*). This gene belongs to the family of zinc finger proteins. Under physiological conditions, the GLI family proteins play an important role in embryonic development. Abnormal operation of these genes causes development defects, eg: Mutations in the GLI2 gene cause defects in the development of the skeleton. In addition, GLI2 has been shown to play a large role in regulating BMP-2 protein expression during osteoblast differentiation (67). The presented research results confirm the relationship between the expression of GLI2 and BMP-4. During the differentiation of GCs towards osteoblasts in long-term *in vitro* culture, GLI2 may interact with BMP-4 in a positive manner (Figs. 2 and 3).

IGFBP-5 (*Insulin-like growth factor-binding protein 5*) is another factor involved in the process of osteoblast formation. This substance is detected during osteoblastogenesis. It is most intensively released during the first days of *in vitro* culture before mature osteoblasts arise. It is known that the amount of this factor decreases during long-term *in vitro* culture (68). In addition, IGFBP-5 produced by osteoblasts stimulates osteoclastogenesis and thus acts as an osteoblast-osteoclast coupling agent (69). Liu and Ling (70) proved that the rat ovary produces 5 types of IGFBP, including IGFBP-5 in GCs of atretic preantral follicles. In addition, the presented study results suggest that IGFBP5 interacts with the SPP1 gene described above (Fig. 2).

According to the results presented, growth factors such as BMP-4 or IGFBP5 interact with genes that, in addition to their role in the ovary, also play key roles in osteogenesis. Therefore, these factors may be potential regulators of the expression of the above genes, as well as the process of GC differentiation towards osteoblasts. As already mentioned, these genes play a key role in the process of osteoblast differentiation from mesenchymal stem cells. The results of the presented studies therefore suggest one of the probable ways of osteo-differentiation of GCs.

Another gene that is expressed by GCs after 30 days of *in vitro* culture is TWIST (*Transcription Factor TWIST*). Under physiological conditions, this gene is expressed in cells that express RUNX2 expression. As mentioned earlier, the RUNX2 gene is involved in the process of osteoblast formation. The basic function of TWIST is the inhibition of the RUNX2 function during skeletogenesis (71). The TWIST expression can, therefore, explain the lack of RUNX2 expression in the presented GCs after 30 days of culture.

Osteoblast differentiation from stem cells is possible thanks to CTHRC1. Wang *et al* (72) indicated that this gene, under physiological conditions, is involved in bone remodelling, and also shows the presence in osteocytes, bone matrix and periodontal ligament cells in rat. In presented research CTHRC1 has been activated during long-term *in vitro* culture of GCs.

In addition to gene expression changes observed during the course of the long-term *in vitro* culture, we have also observed significant changes of morphology. These changes, documented in Fig. 5, show that in the first days of culture the cells assume star-like shape, followed by their elongation and transition into fibroblast like shape in the later stages of culture. Similar results can be found in literature (22,44). This fact can be associated with the assumed loss of granulosa specific gene expression, caused by the absence of the physiological extracellular environment, and assumption of new, culture-specific phenotype. This process is also accompanied by upregulation of expression of non-granulosa specific growth factors (such as BMP4 and IGFBP5), which may further explain the assumption of new morphology.

Summing up the conducted research, it can be concluded that there is a lot of evidence for the possibility of GC differentiation towards osteoblasts. Obtaining stable cultures of differentiated osteoblasts derived from GCs may find wide application in the treatment of skeletal disorders. However, it needs to be noted that this is an entry level transcriptomic study, which only accounts for the gene changes observed in the granulosa cell culture and refers them to the available literature. Despite that, the presented research is an excellent "signpost" for further, more detailed studies, possibly including comparisons to osteoblasts cultured in similar conditions, or based on proteomic approaches (which are much better translatable to *in vivo* knowledge). Detailed analysis of pathways involved in the differentiation of GCs towards osteoblasts is required.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MBrą provided resources (provision of study materials and patients), designed the experiments and methodology, and wrote the original draft of the manuscript. WK performed the investigation (conducted the research and investigation process, performed the experiments, or data/evidence collection), developed the methodology, and wrote the original draft. PC developed the software, created and presented the published work, wrote the initial draft, and performed the formal analysis and visualization. KO conducted the experiments, acquired data and wrote the manuscript. JBT conducted the experiments, acquired data and wrote the manuscript. MJ designed the methodology and created the models. LP designed the study and revised the medical methodology. MBru contributed to medical procedure design and approved the final draft of the manuscript. MN supervised the study, designed the experiments and provided editorial supervision. MZ revised the methodology, analyzed data and approved the final draft of the manuscript. BK conceived the study, contributed to project administration, acted as the senior author and provided major assistance during the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study has been approved with resolution 558/17 by Poznan University of Medical Sciences Bioethical Committee. All participants gave their written informed consent for use of their material in research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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