Evaluation of changes arising in the pig mesenchymal stromal cells transcriptome following cryopreservation and Trichostatin A treatment

Artur Gurgul¹, Joanna Romanek², Klaudia Pawlina-Tyszko¹, Tomasz Szmatoła¹, Jolanta Opiela²*

¹ National Research Institute of Animal Production, Department of Genomics and Molecular Biology, Balice, Poland, ² National Research Institute of Animal Production, Department of Animal Reproduction Biotechnology, Balice, Poland

* jolanta opiela@izo.krakow.pl

Abstract

Cryopreservation is an important procedure in maintenance and clinical applications of mesenchymal stem/stromal cells (MSCs). Although the methods of cell freezing using various cryoprotectants are well developed and allow preserving structurally intact living cells, the freezing process can be considered as a severe cellular stress associated with ice formation, osmotic damage, cryoprotectants migration/cytotoxicity or rapid cell shrinkage. The cellular response to freezing stress is aimed at the restoring of homeostasis and repair of cell damage and is crucial for cell viability. In this study we evaluated the changes arising in the pig mesenchymal stromal cell transcriptome following cryopreservation and showed the vast alterations in cell transcriptional activity (5,575 genes with altered expression) suggesting the engagement in post-thawing cell recovery of processes connected with cell membrane tension regulation, membrane damage repair, cell shape maintenance, mitochondria-connected energy homeostasis and apoptosis mediation. We also evaluated the effect of known gene expression stimulator—Trichostatin A (TSA) on the frozen/thawed cells transcriptome and showed that TSA is able to counteract to a certain extent transcriptome alterations, however, its specificity and advantages for cell recovery after cryopreservation require further studies.

Introduction

Mesenchymal stem cells (MSCs) are highly attractive for tissue engineering and clinical applications because of their inherent regenerative capacity, high proliferation potential, immunomodulatory activity, ability to differentiate into different cell lineages and low immunogenicity [1]. In most methodologies, MSCs are enriched from bone marrow aspirates by density gradient centrifugation [2], but their amount usually is insufficient for further procedures. Isolation protocols resulting in high initial cell counts are available and desirable for one-step
procedures in regenerative medicine [3, 4], however, they are cost and time-consuming. This makes *in vitro* expansion of undifferentiated MSCs an indispensable procedure for both scientific and application purposes. *In vitro* expansion, however, carries the risk of contamination by pathogens or malignant transformation and in addition, the multilineage differentiation capacity of stem cells may be lost during long-term expansion [5]. In various application procedures, cryopreservation plays an important role in obtaining off-the-shelf availability for cells. It also separates cell culture from application and prepares cells for long distance transportation and long term storage.

Cryopreserved cells are likely to be the main cell source for tissue engineering and stem cell therapy [6, 7]. Thus, development of technologies which allow storing and banking of MSCs with minimal loss of cell viability, differentiation capacity and function has been under active investigation. It was found that cryopreservation can affect differentiation ability of stem cells [8, 9] and cause the loss of a variety of pluripotency markers [10, 11], but exact reasons for these changes remain elusive. On the other hand, several studies using MSCs derived from different tissues and cryopreserved with 10% Me₂SO applying slow freezing protocols showed that the frozen MSCs maintained similar phenotypes, cell surface markers and growth rates in comparison to freshly cultured cells [12, 13]. A fast freezing protocol employing vitrification have also been investigated, showing normal proliferation, phenotype and differentiation of MSCs [14, 15]. Nevertheless, cryopreservation and cell storage can be considered as environmental stress [16], which can be mediated through one or a combination of different factors, such as cytotoxicity of cryoprotective agents [17], osmotic injury caused by the excursion of a cryoprotective agent upon a freeze-thawing cycle [18], intracellular ice formation during the cooling process [19] and re-crystallization of the intracellular ice during the warming process [20]. Long term storage of cryopreserved human PBMCs resulted in disturbances of transcript levels of 1,367 genes, whose expression after 14 months was affected >3 fold following isolation, cryopreservation and thawing as compared to freshly isolated PBMC [21]. The cryopreservation-induced stress was also described in fibroblasts grown in three-dimensional culture, where it induced a specific cellular stress response involving growth factors [16]. Moreover, it is also assumed that cryopreservation may disturb epigenetic mechanisms associated with cell development and differentiation. In studies investigating cryopreserved zebrafish genital ridges or human spermatozoa a methylation level of some genes was found to be altered after a freezing-thawing procedure. Also the transcript levels of important pluripotency factors like *pou5f1* and *sox2* were found to be altered after cryopreservation [22].

Maintenance of stemness of adult stem cells is to a large extent governed by unique combinations of epigenetic regulators [23]. Epigenetic mechanisms, including histone acetylation/deacetylation, play a crucial role in transcriptional regulation via remodeling of chromatin architecture [24]. Histone deacetylases (HDACs), classified into four classes [25, 26] catalyze a wide spectrum of physiological processes including proliferation, differentiation, apoptosis and cell cycle regulation [27]. One of commonly used culture media factors which was shown to interfere with histones acetylation and stabilize the expression of pluripotent genes is Trichostain A (TSA). TSA is an organic compound that serves as an antifungal antibiotic and selectively inhibits the class I and II mammalian HDACs [28]. It was found that human MSCs treated with a low dosage of TSA *ex vivo* show increased expression of pluripotent genes such as *Oct4, Sox2, Nanaog, Rex-1* and *TERT*. Administration of low concentrations of TSA also significantly suppressed morphological changes in MSCs occurring during culture expansion, increased their proliferation potential and at the same time retained their contact inhibition properties and multipotent differentiation abilities [29].

According to the above, in this study we hypothesized that TSA, through its epigenetic activity, may efficiently counteract the effect of cryopreservation stress in pig bone-marrow
derived MSCs. To verify this hypothesis, we attempted to evaluate changes in MSC gene expression profiles caused by a freezing-thawing procedure and evaluated the result of TSA addition on profile of genes expression in these cells. We additionally performed a quantitative analysis of genes associated with critical biological processes affected either by cryopreservation or TSA administration and evaluated their transcriptional activity in cells harvested at different culture stages including: control fresh MSC, MSC subjected to cryopreservation, TSA-treated MSC and MSC cultured another 24 h after TSA removal.

Methods

Animals

Five outbred Polish Large White (PLW) pigs of both sexes, weighing approximately 20 kg each, were maintained under conventional conditions in the pigsty in the Department of Animal Reproduction Biotechnology at the National Research Institute of Animal Production in Balice, Poland. The veterinary care was provided by the institution. All animal procedures were approved by the Local Animal Care Ethics Committee No. II in Kraków—permission number 1256/2015 in accordance with EU regulations.

Isolation and in vitro culture of pig mesenchymal stromal cells (pMSCs)

The pig mesenchymal stromal cells (MSCs) were isolated as described earlier [30]. In brief, the bone marrow was aspirated from the iliac crests of the animals under general anaesthesia (Thiopental i.v.- 0.01g/kg). The animals after the procedure were provided with analgesics (Pyralgin i.m. -15-50 mg/kg) and anti-inflammatory and antibacterial agents (Betamox i.m.– 0.1 ml/kg). Mononuclear cells were separated by gradient centrifugation at 400 x g for 20 min over a layer of Ficoll-Paque Plus (Stem Cell Technologies; SCT, Canada) and cell suspensions were seeded at a concentration of 1x 10^5 cells/cm^2 per 75 cm^2 culture flask (Corning, USA) containing 17 ml medium comprised of low-glucose Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, Germany) supplemented with 10% foetal bovine serum (FBS; Sigma-Aldrich, Germany) and 1% antibiotic-antimycotic solution (AAS; Sigma-Aldrich, Germany). The cells were incubated at 39˚C and 5% CO_2 in 100% humidified air. Non-adherent cells were removed after the first 24 h. The culture medium was changed every 3 days thereafter. When cells reached about 80–90% confluence, they were detached and digested into single-cell suspensions using 0.25% trypsin-0.01% EDTA solution (Sigma-Aldrich, Germany) and either cryopreserved in 10% Me_2SO/FCS (a detailed description below) or suspended in 100 μl PBS and snap-frozen in liquid nitrogen (LN2) to lyse cells. The snap-frozen MSCs in PBS constituted the non-cryopreserved control group (CTR).

The expression of surface antigens typical for MSCs by flow cytometry and MSC differentiation

The detailed analysis of surface antigens (positive and negative) typical for MSCs by flow cytometry was described in our latest paper [30]. The differentiation of MSCs into osteoblasts and adipocytes was described by Opiela et al. [31].

Cryopreservation and thawing of pMSCs

After dissociation with 0.25% trypsin-EDTA solution, MSCs were washed twice with HEPES supplemented with 10% FBS by centrifugation at 500xg for 5 min, and then 0.5 x number of passaged flasks milliliters of 10% Me_2SO/FCS was added to the washed cells as the freezing
medium. The cells suspended in 0.5 mL of the freezing medium were transferred into 1.0 mL cryovials (Thermo Scientific, Roskilde, Denmark) which were directly transferred to the freezer (-20°C) and stored for 2 hours. Then, the cryovials were quickly dipped into LN2 and stored for 2 weeks before thawing.

The cryopreserved MSCs of five pigs were thawed at 37°C in a water bath with gentle shaking and the cryoprotective solution was removed by gradual dilution with TCM199-HEPES medium supplemented with 10% FBS. The cells were centrifuged at 500×g for 5 min to remove the freezing medium. Then, the cells from each pig were re-suspended in three flasks filled with 17 mL of pMSCs medium (low-glucose DMEM) enriched in 10% foetal bovine serum (FBS), 1% antibiotics and/or 1% Glutamax (Invitrogen, USA) at the density of approx. 1x 10^4 cells/cm^2 for *in vitro* culture. The cells were allowed to attach and TSA was added to the medium of two flasks and incubated for 24 h when the confluency reached 60–70%. After that time, the medium was discarded, the cultures were terminated by trypsinisation as described above and the cells were suspended in 100μl PBS without Ca and Mg followed by snap freezing in LN2 to lyse the cells. The remaining third flask of each pig was further cultured for additional 24 h. By such procedure we obtained three experimental groups, namely: 1. The cryo control group—frozen/thawed MSCs *in vitro* cultured in DMEM supplemented with 5%FCS (Cryo); 2. The TSA group—frozen/thawed MSCs *in vitro* cultured in DMEM supplemented with 5%FCS and 50nM TSA for 24 h (TSA); 3. The TSA >24 group—frozen/thawed MSCs *in vitro* cultured in DMEM supplemented with 5%FCS and 50nM TSA for 24 h followed by 24 h of *in vitro* culture in DMEM/5%FCS without TSA (TSA >24). The cultures were terminated by trypsinisation as described above and the cells were suspended in 100μl PBS without Ca and Mg followed by snap freezing in LN2 to lyse the cells. The cryovials were stored in LN2 for 2 to 4 weeks before a RNA-seq analysis.

### Analysis of frozen/thawed MSC specific markers (CD73, CD90, CD105) by Western-blotting

MSCs of three different pigs, cryopreserved in 10% Me2SO/FCS when the round shape colonies were already formed and stored few weeks in liquid nitrogen were thawed and re-plated into T75 culture flasks. The cells were *in vitro* cultured in DMEM supplemented with 5% FCS till reaching 70% confluence. Then, each biological sample was divided into three treatments: 1. *in vitro* culture in DMEM supplemented with 5% FCS (Cryo); 2. *in vitro* culture in DMEM supplemented with 5%FCS and 50nM TSA (TSA); 3. *in vitro* culture in DMEM supplemented with 5%FCS and 50nM TSA for 24 h followed by 24 h in DMEM/5% FCS (TSA >24h). On the day of the experiment, samples were solubilized in tissue protein extraction reagent T-PER (Thermo Fisher Scientific) containing a protease inhibitor cocktail (539134, Merck, Warsaw, Poland). Samples were sonicated for 10 sec in an ultrasonic cell disruptor (TORBEO, 36810; Cole—Parmer, Vernon Hills, USA) after 5 min incubation. The denatured samples were separated by electrophoresis on a discontinuous SDS gel system consisting of 4% polyacrylamide stacking and 12% separating components. Then, proteins were transferred from the gels onto 0.22 mm PVDF membranes in 10 mM CAPS containing 10% methanol using a semidy electroblotting apparatus (Bio-Rad, Warszawa, Poland) at 50 V for 50 min. The membranes were blocked with TBS-T (25 mM Tris, 0.5 M NaCl, pH 7.5 + 0.1% Tween 20) blocking buffer and supplemented with 0.2% (w/v) skim milk powder. Blocking, washing and incubations with primary and secondary antibodies were performed at room temperature in the Snap id system (Merck). The blocked membranes were incubated for 10 min at room temperature with primary antibodies for CD73, CD90 and CD105 (Santa Cruz Biotechnology, Inc, USA). The description of antibodies and the used concentrations are provided in Table 1.
The membranes were washed four times in TBS-T and incubated for 10 min at room temperature with the appropriate secondary antibody (Santa Cruz Biotechnology, Inc, USA) conjugated to horseradish peroxidase (HRP) at a specific concentration. The membranes were washed again three times with TBS-T. Subsequently, the proteins were visualized using a chemiluminescence kit (Pierce Biotechnology, Inc. Rockford, IL, USA) according to the manufacturer’s instructions (Vilber Lourmat, Marne La Vallée, France). The images were scanned, and the optical density (OD) of the bands was measured using Fusion Solo.6S.WL. For re-probing with actin as the loading control, the membrane was washed in stripping buffer (100 mM 2-mercaptoethanol, 20% SDS, 62.5 mM Tris—HCl, pH 6.7) at 56˚C for 60 min to strip off the bound antibody. After washing in TBS-T and blocking, the membrane was re-probed with the goat polyclonal anti-actin antibody (sc–1615, Santa Cruz Biotechnology, Inc, USA) (0.2 μg/mL) for 10 min at room temperature. The secondary anti-goat HRP-labelled antibody (sc–2020, Santa Cruz Biotechnology, Inc, USA) (0.1 μg/mL) was incubated for 10 min at room temperature. Actin was visualized as previously described by chemiluminescent reaction. The images were scanned, and the optical density (OD) of the bands was measured using Fusion Solo.6S.WL (Vilber Lourmat, Marne La Vallée, France). The ratio of the OD of analyzed proteins relative to actin is presented in the form of bar charts. The significant differences in protein levels were assessed by Tukey’s post–hoc one–way ANOVA.

Library preparation and transcriptome sequencing
Total RNA was purified from harvested cells using Direct-zol™ RNA MiniPrep Kit (Zymo Research, CA, USA) and controlled in terms of quality using Agilent TapeStation2200 system. Only samples with RNA Integrity Number (RIN) above 8 were used for further analysis. In total, 500 ng of RNA was used for library construction with the TruSeq RNA Sample Prep v2 kit (Illumina, San Diego, CA). Standard library preparation steps including: mRNA selection, fragmentation, cDNA synthesis, end repair, adenylation, indexed adapters ligation and amplification were followed by a qualitative evaluation (Agilent TapeStation 2200) and quantitation (Qubit, Thermo Fisher Scientific). The validated, normalized and pooled libraries were ultimately sequenced in a single 50-bp indexed run (1 x 50 bp) on the HiScanSQ system using TruSeq SBSv3 Sequencing kit (Illumina). Raw reads were deposited in NCBI Sequence Read Archive (SRA) database under BioProject number PRJNA395970.

Data analysis
The obtained raw reads were controlled for quality using FastQC software and filtered/trimmed with Flexbar software [32]. The filtering allowed removing reads with phred quality score below 20 and read length below 30nt after adapter trimming. The filtered reads were mapped against the Sus scrofa10.2 genome with the TopHat2 software [33] set to single-end mode and Bowtie “sensitive” option. The reads mapped to exons were counted using HTSeq software in ‘Union’ mode [34], providing Ensembl GTF annotation file v10.2.87. Data
normalization and a differential expression analysis were performed using DESeq2 software [35] in pairwise comparisons among the study groups. The quality of the RNA-Seq read mapping was controlled using RseQC software [36]. Differences in gene expression profiles among the groups were evaluated using principal component analysis (PCA) and hierarchical clustering. The adjusted p-value < 0.05 (after FDR correction using the Benjamini-Hochberg procedure, q-value) was used as a cutoff for differentially expressed (DE) genes. To allow direct comparison of gene-specific read counts when analyzing separate genes (pluripotency, differentiation markers and others), raw reads count tables for individual samples were normalized together in an additional step for all the study groups using DESeq2.

The detected DE genes were evaluated in terms of their biological functions and modulated pathways with respect to gene ontology (GO) terms in PANTHER Classification System [37]. Additional GO and KEGG pathway analyses were performed using KOBAS 3.0 web server [38] or WebGestalt toolkit (WEB-based GEne SeT AnaLysis Toolkit [39]). Only processes with similar biological significance found to be overrepresented by at least two of these algorithms were considered. All the overrepresentation/enrichment tests were performed with respect to all known genes and with a correction for multiple testing.

qPCR validation
Validation of the obtained results was carried out with the use of RT-qPCR method. First, primers specific for seven chosen genes including five pluripotency marker genes with low, medium and high expression levels (KLF4, FGF2, LIF, TERT, NANOG), one neurogenic differentiation marker (NSE) and one highly expressed SOX family member (SOX9) were designed (S1 File). Then, High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific) was used to reverse transcribe selected RNA samples (with sufficient amount of RNA left after the RNA-Seq analysis). AmpliQ 5x HOT EvaGreen® qPCR Mix Plus (ROX) (Novazym) was used to run qPCR reactions according to the manufacturer instructions. The reactions were run in triplicates for each sample including melting curve analysis on QuantStudio™ 7 Flex Real-Time PCR System (Thermo Fisher Scientific). To determine endogenous control that is a gene with the most stable expression, obtained data were analyzed using NormFinder software [40]. Next, ΔΔCt method corrected for reaction efficiency (E) calculated on the basis of standard curves was applied to quantify relative expression levels [41]. The RNA-Seq results comparison with qPCR was based on evaluation of correlation coefficients between expression level of each gene across samples determined with both methods and across genes (averaged expression per gene).

Results

The expression of surface markers typical for MSCs estimated by flow-cytometry in MSCs not subjected to freezing

We proved by flow cytometry the mesenchymal origin of the porcine bone-marrow derived cells. The results are already published by Gurgul et al. [30]. The analysis was performed on isolated MSCs and TSA-modulated and unmodulated MSCs not subjected to freezing/thawing procedure. Moreover, we proved the mesenchymal origin of the porcine bone-marrow derived cells by performing their differentiation into osteoblasts and adipocytes [31].

The expression of positive surface markers typical for MSCs estimated by Western-blotting in frozen/thawed MSCs

Frozen control MSCs (cryo group) and MSCs subjected to TSA following cryopreservation (TSA and TSA > 24h) were evaluated for the expression of CD73, CD90 and CD105 proteins...
by SDS-PAGE and immunoblotting with polyclonal antibodies anti-CD73, anti-CD90 and anti-CD105 (Figs 1 and 2).

The cells of all the analyzed groups expressed the typical MSC markers confirming their multipotent character. Moreover, no significant differences were noted in CD73, CD90 and CD105 proteins expression in MSCs from three treatment groups implying that TSA has neither positive nor negative impact on these markers’ expression (Figs 1 and 2).

![Fig 1. Densitometry of CD73, CD90 and CD105 proteins expression in MSCs from three different treatments: 1-cryo; 2- TSA; 3- TSA >24. Cryo–cryopreserved, not treated cells
TSA–cells treated with TSA for 24h
TSA >24h –cells treated with TSA for 24h and further cultured following TSA removal.](https://doi.org/10.1371/journal.pone.0192147.g001)

![Fig 2. Exemplary protein bands after the Western blot analysis of CD105, CD90, CD73 and actin in MSCs from three different treatments: Cryo, TSA and TSA >24. Cell lysates were subjected to SDS-PAGE and analyzed by Western blotting. The membrane was probed with an antibody against CD73. The same blot was reprobed with an antibody against actin. The same procedure was repeated for estimating CD90 and CD105. Cryo–cryopreserved, not treated cells
TSA–cells treated with TSA for 24h
TSA >24h –cells treated with TSA for 24h and further cultured following TSA removal.](https://doi.org/10.1371/journal.pone.0192147.g002)
Sequencing reads statistics

The RNA-Seq libraries belonging to four study groups encompassing four to five MSC cultures obtained from five different pigs were sequenced in single-end 50-bp run, which resulted in about 564.4 million of raw reads. The number of reads generated per sample ranged from 24.0 to 46.6 M with a mean for separate groups ranging from 27.6 to 34.1 M. On average, 84% of filtered reads were successfully mapped against the reference genome and 13.3% of these reads had multiple mappings. The comparable number of expressed genes (with mean normalized read counts > 1) was detected for the control, frozen-thawed and cultured after TSA removal MSCs (about 13.4 thousand), with a slightly higher number of expressed genes observed in the frozen TSA-treated cells (14.2 thousand) (Table 2). The analysis of expression profiles of all analyzed cultures using hierarchical clustering with genes showing the highest variation among the study groups revealed a general expression profile similarity within the groups and visible inter-group differentiation. Expression profiles of two cultures prolonged after TSA removal (TSA > 24h) showed similar, but still distinct, profile of expression in comparison to the cells harvested after 24h of TSA treatment (Fig 3).

Table 2. Sequencing reads and mapping statistics for all analyzed samples.

| Sample | Group | Number of raw reads | Number of reads after filtering | % of mapped reads | % of reads with multiple mappings | Number of reads uniquely mapped to genes | % of reads uniquely mapped to genes in regard to all reads mapped | Number of detected genes (normalized read count > 1) |
|--------|-------|---------------------|---------------------------------|------------------|-----------------------------------|------------------------------------------|-----------------------------------------------------------------|-------------------------------------------------|
| 47     | Control | 33,527,496          | 33,232,716                      | 87.0             | 14.6                              | 19,281,625                              | 66.7                                                            | 13,402                                          |
| 52     | Cryo   | 29,337,818          | 29,051,179                      | 85.8             | 14.5                              | 16,554,887                              | 66.4                                                            | 14,074                                          |
| 54     |       | 32,501,711          | 32,245,946                      | 86.5             | 14.6                              | 18,459,014                              | 66.2                                                            | 14,246                                          |
| 55     |       | 36,884,314          | 36,708,672                      | 86.9             | 14.7                              | 21,185,195                              | 66.4                                                            | 13,547                                          |
| All (mean) |       | 33,062,835         | 32,809,628                      | 86.6             | 14.6                              | 18,870,180                              | 66.4                                                            | 13,817                                          |
| 9      |       | 39,811,022          | 39,383,308                      | 86.5             | 12.3                              | 21,009,157                              | 61.7                                                            | 13,537                                          |
| 21     | Cryo   | 31,911,754          | 31,657,966                      | 86.4             | 12.4                              | 17,096,115                              | 62.5                                                            | 13,776                                          |
| 23     |       | 26,988,514          | 26,751,854                      | 83.8             | 14.3                              | 14,613,012                              | 65.2                                                            | 13,894                                          |
| 26     |       | 28,433,528          | 28,101,216                      | 84.9             | 13.2                              | 15,698,595                              | 65.8                                                            | 13,950                                          |
| 37     |       | 24,011,188          | 23,860,067                      | 78.8             | 13.3                              | 11,877,827                              | 67.5                                                            | 13,682                                          |
| All (mean) |       | 30,231,201         | 29,950,882                      | 83.1             | 13.1                              | 16,058,941                              | 64.5                                                            | 13,768                                          |
| 19     | TSA    | 32,611,524          | 32,221,390                      | 86.9             | 11.5                              | 17,572,194                              | 62.8                                                            | 14,406                                          |
| 27     |       | 26,944,001          | 26,583,205                      | 83.1             | 12.3                              | 14,832,174                              | 67.1                                                            | 14,539                                          |
| 28     |       | 26,311,428          | 25,950,421                      | 81.2             | 12.4                              | 14,101,883                              | 66.9                                                            | 14,575                                          |
| 32     |       | 24,388,215          | 24,162,907                      | 80.6             | 12.6                              | 13,234,780                              | 68.0                                                            | 13,329                                          |
| All (mean) |       | 27,563,792         | 27,229,481                      | 83.0             | 12.2                              | 14,935,258                              | 66.2                                                            | 14,212                                          |
| 4      | TSA > 24h | 34,013,910         | 33,511,242                      | 85.8             | 13.5                              | 17,274,048                              | 60.0                                                            | 13,536                                          |
| 5      |       | 37,985,222          | 37,506,718                      | 86.0             | 13.6                              | 19,971,007                              | 61.9                                                            | 13,653                                          |
| 10     |       | 46,651,711          | 46,251,409                      | 86.0             | 13.3                              | 25,326,901                              | 63.7                                                            | 14,036                                          |
| 25     |       | 26,118,944          | 25,933,779                      | 80.5             | 12.9                              | 14,034,619                              | 63.2                                                            | 14,101                                          |
| 38     |       | 25,941,128          | 25,863,891                      | 81.0             | 12.5                              | 14,025,411                              | 66.9                                                            | 14,150                                          |
| All (mean) |       | 34,142,183         | 33,813,408                      | 83.9             | 13.2                              | 18,126,397                              | 63.1                                                            | 13,895                                          |
| All (mean) |       | 31,354,079         | 31,054,327                      | 84.0             | 13.3                              | 17,008,247                              | 64.9                                                            | 13,913                                          |

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Effect of cryopreservation on the pig MSCs transcriptome

The effect of a freezing-thawing procedure on the pig MSC transcriptome was evaluated based on cells obtained from five cryopreserved and four control cultures. The comparison of general expression profiles of the frozen and control cells across the genome (measured as a correlation coefficient between mean expression level of all genes in each group), suggested that the cryopreservation introduced significant changes in behavior of several genes ($r = 0.837; p < 0.01$). Further differential gene expression analysis showed extensive alterations in the MSC transcriptome manifesting by significant up- or downregulation of 5,575 different genes (with genome-wide $q < 0.05$). Almost equal number of the genes was up- and downregulated in the frozen cells with the average absolute log$_2$ fold change of 1.09 ($\pm 0.642$) (Fig 4).

To minimize the number of DE genes and allow a more detailed analysis of their biological functions, the top 1000 DE genes with the lowest adjusted $p$-values were selected ($p < 1.0E-05$) (S2 File). Among these genes, 391 were upregulated with the mean log$_2$FC of 1.54 and the remaining ones were downregulated with a similar order of magnitude of log$_2$FC (-1.61). The analysis of physiological implications of the genes aiming at the identification of major affected cellular processes, showed that the upregulated genes significantly enriched molecular functions associated with e.g. cytoskeletal protein binding or actin binding and were mainly related to biological processes involved in cell adhesion, biological adhesion, cellular component...
organization and regulation of cell communication. The upregulated genes also enriched several biological processes responsible for negative regulation of general biological or cellular processes and positive regulation of intracellular protein transport, protein import into nucleus and nucleocytoplasmic transport. Cellular components enriched with the upregulated genes encompassed mainly integral components of membrane and those localized in cell periphery, but also adherens and anchoring junctions, focal adhesions or stress fibers (Table 3). The analysis of Panther, KEGG and Reactome pathways showed that the upregulated genes enriched pathways that are involved in integrin signaling (P00034), Wnt signaling (P00057) tight junction (ssc04530), regulation of actin cytoskeleton (ssc04810), inflammatory mediated regulation of TRP channels (ssc04750) and pathways in cancer (ssc05200).

Molecular functions enriched with the genes downregulated after cryopreservation included mainly those associated with translation (e.g. structural constituent of ribosomes, RNA/poly(A) RNA binding) or energy homeostasis (e.g.: electron carrier activity, NADH dehydrogenase activity, cytochrome-c oxidase activity, ATP binding and general oxidoreductase activity). The genes enriched molecular function-related biological processes, including inter alia: translation, gene expression, ribosome biogenesis, nitrogen compound metabolic processes, hydrogen ion transmembrane transport, ATP metabolic process, oxidative phosphorylation, respiratory electron transport chain or purine ribonucleotide metabolic processes. The top enriched cellular components in which the downregulated genes manifest their functions, involved mainly: mitochondrion, mitochondrial envelope, mitochondrial inner membrane, ribosome, intracellular ribonucleoprotein complex, and cytoplasmic cell part. The genes downregulated after cryopreservation enriched several pathways of which the most interesting seem to be: ribosome (ssc03010), oxidative phosphorylation (ssc00190), respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by

Fig 4. Log₂ fold change and the mean expression level for all genes in the studied MSCs. Significantly differentially expressed genes are marked in red, A- in comparison to preceding treatment; The genes expression level was compared between the frozen and non-frozen control, the TSA treated cells and frozen control and the cells further cultured following TSA removal vs. the TSA treated cells; B- in comparison to the fresh (non-frozen) MSCs.

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uncoupling proteins (R-SSC-163200), nonsense-mediated decay (NMD) (R-SSC-927802), mitochondrial translation (R-SSC-5368287) or translation initiation complex formation (R-SSC-72649) pathways (Table 4).

Effect of TSA administration on the transcriptome of cryopreserved MSCs

The culture of cryopreserved MSCs with 50mM TSA for 24 h resulted in significant changes in transcript levels of lower number of genes than in the case of cryopreservation itself, nevertheless, as many as 2759 genes were affected, of which about 58% were upregulated. The absolute average log₂ fold change for the upregulated genes was about two times higher than for the downregulated (1.61 vs. 0.81) (Fig 4). The correlation coefficient between the mean gene

### Table 3. Summary of biological processes, molecular functions and cellular components affected by genes upregulated in the post-thawing MSC culture.

| GO | term ID | Description                              | log10 p-value |
|----|---------|------------------------------------------|--------------|
| BP | GO:0016043 | cellular component organization           | -3.9172      |
|    | GO:00051606 | detection of stimulus                     | -3.7212      |
|    | GO:0007606  | sensory perception of chemical stimulus   | -3.3696      |
|    | GO:0071840  | cellular component organization or biogenesis | -3.0975 |
|    | GO:0007043  | cell-cell junction assembly              | -2.5901      |
|    | GO:0048519  | negative regulation of biological process | -2.0277      |
|    | GO:0048523  | negative regulation of cellular process   | -2.0277      |
|    | GO:0032879  | regulation of localization                | -2.0277      |
|    | GO:0090136  | epithelial cell-cell adhesion             | -2.0277      |
|    | GO:0048518  | positive regulation of biological process | -2.0214      |
|    | GO:0045444  | fat cell differentiation                  | -1.7194      |
|    | GO:0051049  | regulation of transport                   | -1.71        |
|    | GO:0034330  | cell junction organization                | -1.6216      |
|    | GO:0022610  | biological adhesion                       | -1.5352      |
|    | GO:0010646  | regulation of cell communication           | -1.5287      |
|    | GO:0023051  | regulation of signaling                   | -1.3768      |
| MF | GO:0005515  | protein binding                           | -10.0132     |
|    | GO:0005488  | binding                                   | -5.2118      |
|    | GO:0019899  | enzyme binding                            | -4.0635      |
|    | GO:0005509  | calcium ion binding                       | -3.2118      |
| CC | GO:0031982  | vesicle                                   | -2.6108      |
|    | GO:0071944  | cell periphery                            | -2.3412      |
|    | GO:0012505  | endomembrane system                       | -2.1791      |
|    | GO:0043226  | organelle                                 | -2.0804      |
|    | GO:0030054  | cell junction                             | -1.8601      |
|    | GO:0043227  | membrane-bounded organelle                | -1.7595      |
|    | GO:0005925  | focal adhesion                            | -1.7061      |
|    | GO:0005886  | plasma membrane                           | -1.7061      |
|    | GO:0005575  | cellular component                        | -1.6882      |
|    | GO:0044444  | cytoplasmic part                          | -1.6778      |
|    | GO:0001725  | stress fiber                              | -1.5009      |
|    | GO:0005856  | cytoskeleton                              | -1.4787      |

Numerous enriched biological processes were analyzed with Revigo system to summarize them by removing redundant GO terms. Not all detected GO categories are displayed.

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Table 4. Summarized biological processes, molecular functions and cellular components affected by genes downregulated in the post-thawing MSC culture.

| GO | term ID   | description                                               | Downregulated | log10 p-value |
|----|-----------|-----------------------------------------------------------|---------------|---------------|
| BP | GO:0006807 | nitrogen compound metabolic process                       | -9.8539       |
|    | GO:0010467 | gene expression                                            | -9.2684       |
|    | GO:1901564 | organonitrogen compound metabolic process                 | -8.8477       |
|    | GO:0006518 | peptide metabolic process                                 | -8.2034       |
|    | GO:0044271 | cellular nitrogen compound biosynthetic process           | -7.9547       |
|    | GO:0043603 | cellular amide metabolic process                          | -7.8697       |
|    | GO:0006412 | translation                                                | -7.8297       |
|    | GO:0007680 | sensory perception                                         | -7.5867       |
|    | GO:1901566 | organonitrogen compound biosynthetic process              | -7.1337       |
|    | GO:0051606 | detection of stimulus                                     | -7.0013       |
|    | GO:0007186 | G-protein coupled receptor signaling pathway              | -6.8665       |
|    | GO:1902600 | hydrogen ion transmembrane transport                      | -6.7905       |
|    | GO:0009593 | detection of chemical stimulus                             | -6.1824       |
|    | GO:0007154 | cell communication                                         | -5.8097       |
|    | GO:0009058 | biosynthetic process                                       | -5.7825       |
|    | GO:0042254 | ribosome biogenesis                                        | -5.6126       |
|    | GO:0032501 | multicellular organismal process                           | -5.5591       |
|    | GO:0023052 | signaling                                                  | -5.3768       |
|    | GO:0044237 | cellular metabolic process                                 | -4.8539       |
|    | GO:0022900 | electron transport chain                                   | -4.6778       |
|    | GO:0008152 | metabolic process                                          | -3.8539       |
|    | GO:0016072 | rRNA metabolic process                                     | -3.6576       |
|    | GO:0009145 | nucleoside triphosphate biosynthetic process              | -3.3969       |
|    | GO:0050806 | nucleobase-containing small molecule metabolic process     | -3.0269       |
|    | GO:0006888 | ER to Golgi vesicle-mediated transport                     | -2.7011       |
|    | GO:0065007 | biological regulation                                      | -2.5969       |
|    | GO:0071704 | organic substance metabolic process                        | -2.475        |
|    | GO:0006123 | mitochondrial electron transport, cytochrome c to oxygen   | -2.3582       |
|    | GO:0046483 | heterocycle metabolic process                              | -2.0159       |
|    | GO:0006091 | generation of precursor metabolites and energy             | -1.8725       |
|    | GO:0002181 | cytoplasmic translation                                    | -1.7457       |
|    | GO:0050896 | response to stimulus                                      | -1.7235       |
|    | GO:0019637 | organophosphate metabolic process                          | -1.6281       |
|    | GO:0006725 | cellular aromatic compound metabolic process               | -1.5482       |
|    | GO:0044238 | primary metabolic process                                  | -1.5072       |

| MF  | GO:0003735 | structural constituent of ribosome                        | -3.00         |
|     | GO:0004871 | signal transducer activity                                 | -8.0899       |
|     | GO:0060089 | molecular transducer activity                              | -8.0283       |
|     | GO:0005198 | structural molecule activity                                | -7.6737       |
|     | GO:0015078 | hydrogen ion transmembrane transporter activity            | -7.6737       |
|     | GO:0009055 | electron carrier activity                                  | -4.857        |
|     | GO:0016675 | oxidoreductase activity, acting on a heme group of donors  | -4.0862       |
|     | GO:0003723 | RNA binding                                                | -4.0862       |
|     | GO:0015082 | heme-copper terminal oxidase activity                      | -4.0862       |
|     | GO:0003954 | NADH dehydrogenase activity                                | -2.9788       |
|     | GO:0043565 | sequence-specific DNA binding                              | -2.6108       |
|     | GO:0044822 | poly(A) RNA binding                                        | -2.4621       |
|     | GO:0018679 | oxidoreductase activity, acting on diphenols and related substances as donors | -2.4621       |
|     | GO:0018651 | oxidoreductase activity, acting on NAD(P)H                | -2.3958       |
|     | GO:0016491 | oxidoreductase activity                                    | -2.33         |
|     | GO:0003701 | nucleic acid binding transcription factor activity         | -1.8861       |
|     | GO:0003700 | sequence-specific DNA binding transcription factor activity | -1.8861       |
|     | GO:0005524 | ATP binding                                                | -1.6596       |

(Continued)
expression levels in cryopreserved cells and TSA treated cells across the genome was 0.981 (p < 0.01), suggesting extensive changes in gene expression profiles. To analyze in details biological significance of the DE genes and to find the major affected processes, we again focused on the top 1000 genes with the lowest adjusted p-value (< 1.0E-04) (S3 File). Of the genes, 699 were upregulated with the average log2FC of 2.21. The downregulated genes (n = 301) were characterized by a lower fold of change, which was reflected in the value of log2FC equal to -1.01. Biological functions enriched with TSA-stimulated genes were roughly similar to those previously observed on fresh MSCs (Gurgul et al., 2017) and involved inter alia: neuron projection development, single-multicellular organism process, nervous system development, neuron development and general developmental process. These biological processes were convergent with molecular functions and cellular components enriched with the genes which included e.g. ion channel activity, neuron part, synapse part, synapse or integral components of membrane.

The genes downregulated by TSA after 24h culture of cryopreserved cells enriched only very few GO categories, however, they could be connected with the processes like:

### Table 4. (Continued)

| GO      | Downregulated term ID | description                                     | log10 p-value |
|---------|-----------------------|-------------------------------------------------|---------------|
| CC      | GO:0032991            | macromolecular complex                           | -3.00         |
| CC      | GO:0098800            | inner mitochondrial membrane protein complex      | -3.00         |
| CC      | GO:0022626            | cytosolic ribosome                               | -3.00         |
| CC      | GO:0044444            | cytoplasm                                        | -36.5638      |
| CC      | GO:003537             | cytoplasm                                        | -36.2299      |
| CC      | GO:0043226            | organelle                                        | -33.466       |
| CC      | GO:0005622            | intracellular                                    | -30.1965      |
| CC      | GO:0044464            | cell part                                        | -13.4685      |
| CC      | GO:0005623            | cell                                             | -13.1965      |
| CC      | GO:0031975            | envelope                                         | -11.8894      |
| CC      | GO:0098796            | membrane protein complex                         | -11.341       |
| CC      | GO:0070469            | respiratory chain                                | -9.0862       |
| CC      | GO:0005829            | cytosol                                          | -7.9101       |
| CC      | GO:0005575            | cellular_component                               | -5.9914       |
| CC      | GO:0070869            | cytochrome complex                               | -4.6946       |
| CC      | GO:0071944            | cell periphery                                   | -4.4134       |
| CC      | GO:0005886            | plasma membrane                                  | -4.118        |
| CC      | GO:1903561            | extracellular vesicle                            | -4.066        |
| CC      | GO:0097526            | spliceosomal tri-snRNP complex                   | -3.4949       |
| CC      | GO:0003013            | organelar ribosome                               | -2.9252       |
| CC      | GO:0034709            | methylosome                                       | -2.7481       |
| CC      | GO:0031982            | vesicle                                          | -2.5719       |
| CC      | GO:0034719            | SMN-Sm protein complex                            | -2.5119       |
| CC      | GO:0005732            | small nucleolar ribonucleoprotein complex         | -2.5119       |
| CC      | GO:0019773            | proteasome core complex, alpha-subunit complex    | -1.8316       |
| CC      | GO:0031974            | membrane-enclosed lumen                          | -1.8186       |
| CC      | GO:0005793            | endoplasmic reticulum-Golgi intermediate compartment | -1.4973   |
| CC      | GO:0000502            | proteasome complex                               | -1.4973       |
| CC      | GO:0032040            | small-subunit processome                         | -1.3255       |

Numerous enriched biological processes were analyzed with Revigo system to summarize them by removing redundant GO terms. Not all detected GO categories are displayed.

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endoplasmic reticulum functioning, intracellular protein transport, vesicle coat synthesis or G-protein coupled receptor activity.

A comparison of genes differentially expressed after MSCs cryopreservation with those further altered by TSA submission showed 1045 common entries (Fig 5). They included 539 genes initially upregulated by cryopreservation stress of which 330 were reversely regulated by TSA. These genes mainly enriched biological processes associated with hydrogen ion transmembrane transport, oxidation-reduction or single-organism metabolic process. The remaining genes initially upregulated by cryopreservation and further stimulated by TSA treatment enriched only a scarce number of categories of biological processes connected with: cells morphogenesis involved in neuron differentiation and cellular components morphogenesis. Transcripts which were suppressed by cryopreservation and their suppression was deepened by TSA administration belonged to 98 genes with functions enriched in biological processes connected with inter alia: intracellular protein transport, establishment of localization or Golgi vesicle transport. Another set of 408 genes which were suppressed by cryopreservation and further upregulated by TSA addition enriched biological processes associated with e.g. cytokinesis, intracellular protein transport or response to stimulus.

A separate analysis was performed to detect genes whose expression was affected by cryopreservation and then restored to the control level after TSA treatment (i.e. differentially expressed after cryopreservation but not after TSA treatment of cryopreserved cells in comparison to the fresh control cells). The gene list consisted of 2427 entries of which comparable numbers were up- \( (n = 1286) \) and downregulated \( (n = 1141) \) with similar \( \log_2 \) fold of change \( (0.86 \text{ vs. } -0.93) \). The genes which were initially downregulated by cryopreservation and upregulated to the control level by TSA administration or natural cell mechanisms, showed overrepresentation in a wide range of biological processes of which the highest number of detected genes was connected with: metabolism (e.g. regulation of cellular metabolic process; regulation of macromolecule metabolic process; regulation of cellular biosynthetic process), gene expression (e.g. regulation of nucleic acid-templated transcription; regulation of RNA biosynthetic process; regulation of transcription from RNA polymerase II promoter), development.

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**Fig 5.** Venn diagram of differentially expressed genes between A— preceding treatments and B— fresh control MSCs. A— The genes expression level was compared between the frozen and non-frozen control, the TSA treated cells and frozen control and the cells further cultured following TSA removal vs. the TSA treated cells; B— in comparison to the fresh (non-frozen) MSCs. CTR—fresh (not cryopreserved) control Cryo—cryopreserved, not treated cells TSA—cells treated with TSA for 24h TSA>24h—cells treated with TSA for 24h and further cultured following TSA removal.

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Changes in the pig MSCs transcriptome following cryopreservation and TSA treatment

Changes in the expression profile of genes involved in major biological processes associated with cryopreservation and TSA treatment

The identification of major biological processes and pathways affected by cryopreservation and TSA administration allowed us to look closer into changes in expression profiles of differentially expressed genes classified with respect to specific altered processes. Expression levels of the genes were evaluated at all four culture conditions (time points), and involved: control fresh MSCs, MSCs subjected to cryopreservation, TSA-treated MSCs and MSCs cultured another 24h after TSA removal. Based on results from the whole-genome analysis, as major classes of biological processes corresponding to cryopreservation stress we selected those which were found to be significantly enriched and had the highest number of involved genes, namely: adhesion, translation, oxidative stress, ATP synthesis, mitochondrial translation, gene expression and cell communication. The major processes affected by TSA treatment found in this study encompassed neurons differentiation and neuron projection development. Additionally, we considered genes engaged in known cellular responses to cryopreservation stress or malignant transformation, such as: apoptosis, inflammatory response and pathways in cancer. Genes engaged in previously described processes [30] affected by TSA administration, like e.g.: exocytosis and immune system response were also included (Fig 6). In most of these processes, cryopreservation and subsequent TSA administration induced noticeable changes in gene expression profiles involving both up- and downregulation without easy to evaluate pattern. Nevertheless, interesting results were obtained when expression profiles of genes engaged in processes responsible for: energy homeostasis (ATP synthesis, mitochondrial respiratory chain, cytochrome-c oxidase activity and NADH dehydrogenase activity) and mitochondrial translation were analyzed in details. The engaged genes were mostly strongly downregulated by cryopreservation, but subsequent TSA treatment exerted an opposite stimulating effect on most of these genes which ceased after TSA removal. Cryopreservation also visibly stimulated

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Fig 6. Changes in expression level of selected differentially expressed genes with division into major processes affected by cryopreservation and TSA treatment. The normalized read counts for all samples were averaged, log₂ transformed and presented as deviations from the expression level observed in fresh control MSCs.

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transcription of majority of genes engaged in cell communication or pathways in cancer. The succeeding TSA administration also showed some signs of suppression of genes involved in carcinogenesis but further stimulated genes engaged in cell communication. In accordance with our previous findings, the coordinated effect of TSA addition on gene expression manifested mainly through stimulation of genes associated with neurogenesis (neuron projection development, nervous system development, positive/negative regulation of neurons differentiation) and cell communication. This effect, however, ceased after TSA removal (Fig 6).

**Effect of cryopreservation and further TSA treatment on pluripotency/multipotency markers expression in pig MSCs**

To evaluate the effect of applied experimental conditions on the expression level of known pluripotency markers such as FGF2, KLF4, c-MYC, POU5F1, NANOG, SOX2, TERT, WNT3A and ZFP42, normalized counts for each of the genes were compared between the separate treatments. Across all the study groups, with the achieved sequencing depth, virtually no expression was found for POU5F1, NANOG, SOX2, WNT3A genes while a low expression level was detected for TERT and ZFP42. The remaining pluripotency-associated genes had intermediate to high transcript levels with some minor changes introduced by a specific treatment. FGF2 and KLF4 expression was significantly elevated (p < 0.05 at a pointwise level) after cryopreservation and remained increased after TSA administration or TSA removal. c-MYC was characterized by a gradually decreasing expression level with a significant transcript loss after prolonged culture without TSA (p < 0.05). A distinct profile of expression was observed for TERT gene, whose expression was elevated after TSA treatment and downregulated in further culture after TSA removal (Fig 7).

**Validation of the results using RT-qPCR**

The obtained RNA-seq results were validated using an alternative method—RT-qPCR. Among the chosen genes, NANOG gene was characterized by the most stable expression therefore it served as an endogenous control. The correlation coefficient between expression level of individual validated genes across samples was satisfying for four out of six analyzed genes and ranged from 0.30 for KLF gene to 0.94 for FGF2 (with a mean of 0.72). For two other genes (NSE,
A correlation coefficient was low or negative suggesting some results bias. Nevertheless, the comparative analysis of expression levels of genes (averaged expression) estimated using both methods showed a satisfying concordance between the RNA-Seq and qPCR results, which correlated with $r = 0.69$, suggesting no significant bias in relative expression estimation for the analyzed genes (S1 File).

**Discussion**

Cryopreservation is currently a standard procedure in most applications involving *in vitro* cell expansion and their preservation for a further use. Cryopreservation allows the storage of cells for prolonged time periods while maintaining them in adequate condition for use in clinical or research settings. Freezing and storing of cells are also standard procedures in reproductive biotechnology where gametes or embryos are cryopreserved and stored for long time while maintaining their viability and developmental abilities. Conventional cryopreservation systems include slow-freezing and vitrification, both having advantages and disadvantages in terms of cell viability and/or scalability [42]. In this study we used a simplified freezing procedure. Cryovials with cells suspended in a cryoprotectant were stored for 2 h in -20°C and then snap-frozen in liquid nitrogen. We observed that after re-placing in culture the cells maintained their phenotype although part of them did not attach which is a normal observation as around 1/5 of the frozen cells dies during the freezing/thawing procedure [43]. This method of freezing has been routinely performed in our laboratory to freeze somatic cells [44, 45] and mesenchymal stem cells [46] used as donors of nuclei for somatic cell cloning purposes as well as VERO cells used as co-culture cells for bovine and goat embryo *in vitro* culture [47, 48]. Presently the cooling protocol of MSCs from various tissues and species is mostly based on controlled freezing [49] although different approaches of cryopreservation of porcine MSC are rather scarcely described compared to other species. Our experiment, due to extensive results on porcine transcriptome, delivers convincing evidence for the simple 2-step freezing procedure to be effective and efficient. There are reports on successful freezing results in other species where a MSCs cooling protocol was not based on controlled freezing using a freezer: e.g. human MSC [50, 51, 52, 53], monkey MSC [54] and dog MSC [55].

Although the effect of cryopreservation on *in vitro* cultured cell phenotypes was widely studied, research on changes arising in the global cell transcriptome is scarce, especially for non-human cell models. Knowledge on gene expression changes emerging as a result of cryopreservation-induced cell damage or environmental stress may be useful for identification of major cellular processes affected by freezing-thawing procedures and development of more efficient cryopreservation methods or culture additives counteracting cell viability loss. The initial results of this study suggested that cryopreservation induces rather a decrease in global transcriptional activity of cells, thus we assumed that culture additives such as TSA (acting rather as a gene expression stimulator) may counteract transcriptional changes induced by cryopreservation and enable faster cell recovery after cryopreservation stress. In the final results the relative transcript loss after freezing and further culturing turned out not to be as large as expected, however, involved downregulation of more than two thousand genes. So, apart from evaluating the effect of cryopreservation on the pig bone-marrow derived MSC transcriptome we also decided to test the influence of TSA on transcriptional activity of frozen cells.

The results of this study showed that the process of MSC cryopreservation with the applied method induced changes in the expression level of more than five thousand genes in further culture of which almost equal numbers were down- or upregulated. The number of affected genes is difficult to compare with results of other research because comprehensive genome-
wide studies analyzing changes in the gene expression profile after cryopreservation are scarce, especially for in vitro cultured cell lines. One of the available studies showed that the expression of 1,367 genes was altered after 14 months of frozen cells storage with >3 fold change in human PBMCs. Moreover, more than three thousand genes were found to be affected with fold of change >2 in these cells [21]. Another available study also showed a large number of altered genes in frozen-thawed human embryonic stem cells and demonstrated that the number of the upregulated genes reaches a maximal plateau within 24h of culture after thawing, and drops down at 48h. The number of the downregulated genes increases gradually along with the time of culturing [56]. The almost equal number of up- and downregulated genes found in this study conform to the above mentioned results because our sampling of cells was carried out after 24h of post-thawing culture.

To analyze in details cellular processes associated with genes affected by cryopreservation, the number of differentially expressed genes was reduced to an arbitral amount of one thousand genes with the highest statistical significance of differences in the expression level. Although using a fold change as an additional cutoff in DE analysis is a commonly used method for narrowing down gene lists, we decided to rely only on p-value, considering that a differentially expressed at a statistically significant level gene needs to be clearly quantitatively different between the biological groups and relatively consistent within each group of replicates. This allowed us to retain genes with high expression profile uniformity within the groups which may be more characteristic for the applied conditions than changes in the expression level of genes with a high fold change and clear within-group variability [57].

Among the selected genes, 39.1% were upregulated by cryopreservation. The analysis of those genes in terms of their functions, allowed the identification of large number of affected cellular processes of which as major we inferred those associated with: cytoskeleton, adhesion, cellular component organization, regulation of cell communication and intracellular protein transport. The genes were found to manifest their function mainly in cellular component such as integral components of membrane and cell periphery or different types of cell junctions. All these findings suggest that among the major cellular mechanisms affected by cryopreservation and connected with upregulation of genes are those associated with cell membrane functioning, cellular adhesion and cytoskeleton functions. It is clear from the cryobiology theory that cell membrane is under a large load during cooling and freezing processes because of osmotic stress and rapid cell shrinkage [58]. Steponkus and Wiest [59] showed that during cooling the cell shrinkage induces irreversible membrane fusion, hence the effective area of cell membrane is reduced and when returned to isotonic conditions, the tension of cell membrane is considerably increased. Moreover, cells were shown to exhibit a reversible inhibition of shrinkage at some point during a progressive increase in extracellular solute concentration, implying the development of membrane stress [60]. Another study identified such mechanisms of slow-freezing injury as macromolecule denaturation from dehydration or phase transition of membrane lipids [61]. The study of molecular events showed that slow freezing affects a membrane phase transition (liquid crystalline to gel phase), whereas rapid freezing is observed to maintain high conformational disorder of the membranes [62]. The currently applied cryoprotectants reduces these effects, however they generate osmotic stress, both during administration and post-thawing removal. So, the observed alterations in transcription levels of genes associated with cell membrane functions are probably a cellular response to all above mentioned stress factors.

It was also previously proven that cell attachment affects the biophysics of freezing. Experimental data showed that intracellular ice formation and water transport were enhanced in attached cells [62]. Liu and McGrath [63] stated that cell-cell and cell-scaffold interactions alter a cell cryopreservation response and, in turn, cellular structures involved in adhesion and
intercellular contact are targets of cryopreservation-induced damage. The Authors also suggested that focal adhesions and gap junctions may support cell robustness during cryopreservation. Terry et al. [64] showed that attachment efficiency of human hepatocytes was significantly decreased after cryopreservation and gene transcripts including integrins, cadherins, catenins, and matrix metalloproteinases (MMPs) were altered by cryopreservation. All these findings suggest that cell adhesion is an important mechanism of cell viability maintenance after cryopreservation (especially for adherent MSCs) and observed changes in the expression level of genes functionally connected with adhesion and cell junctions are another important cellular response to freezing stress.

Cell adhesions can be described as a functional extension of the actin cytoskeleton [65]. The cellular cytoskeleton may have a significant role in determining post-dehydration and post-thaw cell viability, thanks to its interaction with the cell membrane. The cytoskeleton is linked almost constantly to the cell membrane [66] and is able to modify a membrane structure [67], alter its shape or morphology [68], change the mechanical properties of cells [69] and influence the membrane water transport [70]. Studies in the area of reproductive biotechnology showed that slow freezing and thawing of camel embryos resulted in a modest level of cell death but, on the other hand, more widespread disruption of the actin cytoskeleton [71]. Their findings agree with our results, which showed that the applied freezing method causes upregulation of genes associated with the regulation of the actin cytoskeleton.

The set of genes that were downregulated in post-thawing culture of the studied pig MSCs was in the vast majority associated with translation, ribosome biogenesis and respiratory processes in mitochondria with particular reference to mitochondrial envelope and its inner membrane. It is understood that changes in a gene expression level on the scale observed in this study must entail a cascade of processes responsible for gene expression regulation, purine metabolism, ribosome assembly and translation initiation. Nevertheless, the role of genes connected with mitochondria functions may be at least twofold in the cellular cryopreservation response. First, large amounts of ATP are needed for transcription and translation processes altered after cryopreservation as well as a cytoskeleton response to freezing stress. Second, it was shown that mitochondria are a target of cryopreservation-induced damage [72] or may trigger apoptosis through the release of mitochondrial pro-apoptotic proteins into the cytosol [73]. In the study investigating mitochondrial respiration of DMSO cryopreserved muscle tissue which compared oxygen flux rates with respect to freshly harvested muscles, it was demonstrated that cryopreservation results in cytochrome c loss and potential disruption of the inner mitochondrial membrane [72]. A review of cellular processes occurring in cryopreserved sperm showed that the cryopreservation can induce structural damage to the mitochondria and alter biochemical processes involved in ATP production [74]. Also fragmentation damage to nuclear DNA and a diminution in sperm motility are mainly associated with damage to the structure and metabolic functioning of the mitochondrion after sperm cryopreservation. A no less important role mitochondria can play in apoptotic processes triggered after cryopreservation. It was shown that MSCs are vulnerable to cryopreservation-induced apoptosis due to the activation of apoptosis-related proteins during thawing, however, the exact relationship between cryopreservation and apoptosis is not yet well understood [75]. The complex role of mitochondria in mammalian cell apoptosis came into focus when biochemical studies identified several mitochondrial proteins that are able to activate cellular apoptotic programmes [76, 77]. In physiological conditions, these proteins reside in the intermembrane space of mitochondria and in response to a variety of apoptotic stimuli, they are released to the cytosol and/or the nucleus. They promote apoptosis either by activating caspases and nucleases or by neutralizing cytosolic inhibitors of this process [78]. The observed here coordinated cryopreservation-induced downregulation of genes connected with mitochondria functions suggests that
the mitochondria play an important role in cell recovery after freezing (and their further viability) and may perform various functions depending on cryopreservation success/failure rate and cell fate decisions.

In this study we also evaluated the effect of trichostatin A administration on the transcriptome of MSCs which underwent cryopreservation. In our previous research we found that TSA is rather a gene expression stimulator in fresh MSCs and acts through the complex cellular epigenetic mechanisms [30]. The effect of TSA on frozen cells was largely similar to that observed for non-frozen cells. TSA mainly stimulated genes connected with neuron projection development, single-multicellular organism process, nervous system development, neuron development and general developmental processes and suppressed the expression of genes connected with endoplasmic reticulum functioning, intracellular protein transport, vesicle coat synthesis or G-protein coupled receptor activity. The most interesting results were, however, obtained when expression profiles of genes affected by cryopreservation and engaged in processes responsible for energy homeostasis (ATP synthesis, mitochondrial respiratory chain, cytochrome-c oxidase activity and NADH dehydrogenase activity) and mitochondrial translation were analyzed in details. TSA treatment exerted an opposite to cryopreservation stimulating effect on most of these genes, but this effect was temporary and ceased after TSA removal. This observation suggests that TSA has potential as a culture additive counteracting cryopreservation induced expression alterations, however, this statement needs further proof involving a cell phenotypical and biochemical properties analysis.

The results of this study also showed that applied cryopreservation conditions do not provoke crucial changes in the expression of known pluripotency marker genes. This is in opposition to some studies showing a loss of a variety of pluripotency markers in cryopreserved MSCs [10, 11], however, it is in line with studies showing that after cryopreservation MSCs maintain similar phenotypes, cell surface markers and growth rates in comparison to fresh cells [12, 13].

When it comes to validation of our results with qPCR, some, but in our opinion not significant, discrepancies were found which concerned two out of six analyzed genes. These discrepancies may result from yet still not accomplished annotation of the pig genome, resulting in the lack of information on all alternative mRNA splice variants. This makes design of qPCR primers representative for all transcripts difficult and may result in differences in observed overall expression levels of genes between the two applied methods.

Conclusions

Summarizing, our results show that cryopreservation induces vast alterations in the transcriptional activity of MSCs which implies engagement in post-thawing cell recovery of processes connected with cell membrane tension or damage, cell shape maintenance, transcriptional activity and mitochondria-connected energy homeostasis or apoptosis mediation. Administration of TSA in post-thawing culture is able to a certain extent to counteract these alterations but this effect is transient and its specificity and advantages in frozen cells require further studies.

Supporting information

S1 File. Primers used for qPCR validation (T1) and results of correlation analysis between qPCR and RNA-Seq methods (T2).
(DOCX)
S2 File. The list of the top 1000 genes affected by cryopreservation along with their annotations.
(XLSX)

S3 File. The list of the top 1000 genes affected by TSA administration in the post-thawing culture along with their annotations.
(XLSX)

Author Contributions

Conceptualization: Artur Gurgul, Jolanta Opiela.

Data curation: Artur Gurgul, Tomasz Szmatoła, Jolanta Opiela.

Formal analysis: Joanna Romanek.

Funding acquisition: Artur Gurgul.

Investigation: Artur Gurgul, Jolanta Opiela.

Methodology: Joanna Romanek, Klaudia Pawlina-Tyszko.

Project administration: Jolanta Opiela.

Software: Tomasz Szmatoła.

Supervision: Jolanta Opiela.

Visualization: Tomasz Szmatoła.

Writing – original draft: Artur Gurgul.

Writing – review & editing: Klaudia Pawlina-Tyszko, Jolanta Opiela.

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