INCREASED TRANSCRIPT EXPRESSION LEVELS OF DNA METHYLTRANSFERASES TYPE 1 AND 3A DURING CARDIAC MUSCLE LONG-TERM CELL CULTURE

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Abstract

Heart failure (HF) is one of the main causes of death worldwide. Recent studies reported altered DNA methylation in failing human hearts. This may suggest a role of DNA methylation, most frequently implicated in epigenetic control, in the development of heart failure. Here, employing RT-qPCR, we characterized transcript levels for main DNA methyltransferases (DNMTs), DNMT1, DNMT3A, and DNMT3B, mediate DNA methylation, and they have different functions that complement each other during methylation. All analyzes were performed at different stages of porcine myocardial cell primary culture. In the present study we demonstrated increasing transcript expression levels for all analyzed genes during *in vitro* cultivation. The changes for DNMT1 and DNMT3A seem to be particularly important, where statistically significant changes were observed.

Running title: DNMTs role in cardiac muscle cell culture

Keywords: epigenetics; DNMTs; cardiac muscle; RT-qPCR

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Introduction

Cardiovascular diseases constitute to 49% of deaths in Poland. The last stage of majority of them is chronic heart failure (HF), defined as a syndrome of clinical symptoms, caused by irreversible damage of cardiomyocytes that prevent proper perfusion of the internal organs and leading to multi-organ end-stage failure. Additionally to the efforts to improve the diagnostic methods and increase the effectiveness of treatment with „traditional” approaches, more and more attention is given to the possibility of utilization of the regenerative potential of human organism, which, according to numerous scientific reports, appears to be also noticeable in the heart muscle tissue. Nevertheless, the scientific world still has not created a clear answer about the intrinsic regenerative capacity of the cardiac muscle. On the one hand, the role of cardiac progenitor cells (CPCs) in tissue self-renewal by CM replenishment is shown [1–3], and on the other hand studies demonstrated that the adult heart lacks a predetermined cardiomyocyte-producing stem cell and mammalian cardiac regeneration is not mediated by *de novo* differentiation of endogenous cardiac stem cells in the adult stage [4,5]. Regardless of the above arguments, it seems extremely important to understand the molecular mechanisms that play a pivotal role in the determination of phenotypic variations in the population of myocardial cells.

Epigenetic mechanisms play an essential role in eukaryotic gene regulation by modifying chromatin structure, which in turn modulates gene expression without changes in the DNA sequence [6]. There are few processes that have been most frequently implicated in epigenetic control, nevertheless DNA methylation seems to be the most common epigenetic mechanisms of gene regulation [7]. The methylation of mammalian genomic DNA, referred as addition of a methyl group to the fifth position of the cytosine pyrimidine ring, predominantly at CpG dinucleotides, is catalyzed by DNA methyltransferases (DNMTs) [8]. Three different DNMTs (DNMT1, DNMT3A, and DNMT3B) mediate DNA methylation, and they have different functions that complement each other during methylation [9]. Because it is well established that this epigenetic modification may affect chromatin accessibility, and the DNMTs characteristic in cardiac muscle culture remains poorly understood, thus we decided to focus our research on the DNA methyltransferases mRNA expression levels in cardiac muscle cells *in vitro* culture.

We studied DNMT1, DNMT3A, and DNMT3B transcript levels in different stages of primary *in vitro* cell culture of cardiac muscle obtained from porcine right atrial appendage.

Materials and methods

Animal tissues

Porcine (*Sus scrofa f. domestica*) hearts, delivered on ice in shortest possible time after slaughter, from a local slaughterhouse were the source of cells for *in vitro* culture. For this study, a pubertal crossbred Polish Landrace (PBZ x WBP) gilts, bred on commercial local farm were used. They had a mean age of 155 days (range 140–170 days) and the mean weight were 100 kg (95–120 kg). All the animals were housed under identical conditions and fed the same forage.

Enzymatic dissociation and primary cell culture

The right atrial appendage (right auricle) was extracted from the delivered material, washed in ice-cold PBS solution, to remove the blood and, in the next stage, after the two-step mincing in petri dishes the tissue undergo enzymatic digestion in DMEM + collagenase type II (2mg/mL) solution conducted in 37°C for 40 min. After the end of digestion, the remaining tissue will be separated with nylon strainers of 70µm pore size. The filtrate (containing cells of interest) will be centrifuged (5 min, 200 x g, RT), in order to remove the remaining collagenase from the cell environment. Cell pellet obtained was washed with the PBS buffer and then placed in culture medium (DMEM/F12, Sigma-Aldrich), 20% FBS (Foetal Bovine Serum, Gibco), 10% HS (Horse Serum, Gibco), EGF (20ng/mL; Sigma-Aldrich), 1% P/S. The cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂. The culture medium was changed every three days.

RNA extraction and reverse transcription

Total RNA from all of the samples (both before and after IVM) was isolated according to the method published by Chomczyński and Sacchi [10] employing TRI reagent (Sigma-Aldrich; Merck KGaA). RNA integrity was determined by denaturing agarose gel (2%) electrophoresis, and then, the RNA was quantified by measuring the optical density (OD) at 260 nm (NanoDrop spectrophotometer; Thermo Scientific, Inc.). The RNA samples were re-suspended in 20–40 µl of RNase-free water and stored at –80°C. RNA samples were reverse-transcribed into cDNA using RT2 First Strand kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Then, 500 ng of an RNA sample was used for reverse transcription.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) Analysis

Determination of the transcript levels for DNMT1, DNMT3A and DNMT3B was conducted using a Light Cycler® 96 Real-Time PCR System, Roche Diagnos-

TABLE 1 Oligonucleotide sequences of primers used for RT-qPCR analysis

GENE	PRIMER SEQUENCE (5'-3')		PRODUCT SIZE (BP)
DNMT1	F	GTGAGGACATGCAGCTTTCA	211
	R	AACTTGTTGTCCTCCGTTGG	
DNMT3A	F	CTGAGAAGCCCAAGGTCAG	238
	R	CAGCAGATGGTGCAGTAGGA	
DNMT3B	F	ACAGGTCGGCTCTTCTTTGA	245
	R	AAAGCCCCTCGTTACCTGTT

FIGURE 1 RT-qPCR quantitative relative changes of analyzed DNMTs presented in a form of a bar graph. The graph shows the relative changes in gene expression results for 7, 15 and 30 days of cultivation, in relation to the transcript levels obtained from beginning of our culture (0d). FC was presented in its logarithmic form to provide clear comparability of the results

tics GmbH (Mannheim, Germany) with EvaGreen as a detection dye. Levels of analyzed transcripts were standardized in each sample, in reference to *porphobilinogen deaminase* (PBGD) and *β-actin* (ACTB) as an internal control. For each of the amplification reactions, 1 μL of cDNA solution was mixed with 9 μL of reaction master mix (5 x QUANTUM EvaGreen® MIX (Syngen Biotech Sp. z o.o. Sp. K., Poland) and a specific starter pair). We have used the Primer3 software for primer design (Tab. 1). The exon-exon design method was used as an additional method to avoid the possible amplification of genomic DNA fragments. The primers were also designed using the sequence of several transcript variants of genes of interest available in the Ensembl database. For target cDNA quantification, we have performed rel-

ative quantification with the $2^{-\Delta\Delta C_T}$ method. In order to confirm the specificity of the results, 2% agarose gel electrophoresis of the products was performed.

Statistical analysis

The normality of the observed data distribution was assessed by the Shapiro-Wilk test, followed by the Mann-Whitney U test to identify statistically significant differences between the compared mean values. *p* values <0.05 were considered to be statistically significant. Statistical analyses were performed using the STATISTICA 13 software.

Ethical approval

The research related to animal use has been complied with all the relevant national regulations and

FIGURE 2 Comparison of ΔC_t values for analyzed DNMTs mRNA levels in different stages of culture. All calculations were made in relation to the expression levels for the individual genes at the time of beginning of the culture

institutional policies for the care and use of animals. As the research material is usually disposed of after slaughter, being a remnant by-product, no Ethical Committee approval is needed for this study.

Results

In the present study, employing RT-qPCR to compare the mRNA levels, DNMT1, DNMT3A, and DNMT3B transcripts in primary *in vitro* cardiac muscle cell culture were identified. Nevertheless, the DNMT3B transcript levels were markedly lower compared with DNMT1 and DNMT3A in each of the stages of cultivation ($p < 0.001$ for DNMT1 and DNMT3A). As shown in **figure 1**, where relative quantification with the $2^{-\Delta\Delta C_t}$ method results are visualized, for all analyzed genes we observed increasing expression during cultivation in relation to the transcript levels obtained from starting point (0d) of our culture. However, statistically significant changes were observed for DNMT1 and DNMT3A mRNA levels in 7, 15 and 30 day of cultivation (**Fig. 2**).

Discussion

Despite significant advances in medical therapy and prophylactic strategies, the prognosis of millions of patients with chronic heart failure (HF)

remains poor. HF is an end stage of many cardiac diseases, particularly coronary artery disease (CAD) and cardiomyopathies. Available therapeutic options for HF individuals are still suboptimal, therefore, many hopes are associated with a new therapeutic approaches that uses the intrinsic regenerative potential of the heart tissue. This is why understanding the molecular mechanisms underlying the functioning of the myocardial cell population becomes a key aspect. Epigenetic alternations, including DNA methylation, undoubtedly play a key role in the activity of mammalian cells.

The mammalian DNA methyltransferases family consist of DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L. The studies presented, focus on the analysis of mRNA expression levels for DNMT1, DNMT3A, and DNMT3B, because DNMT3L is primarily restricted to early embryogenesis and DNMT2 functions to methylate RNA [11]. Among group of the DNMTs, two ways for establishment and mitotic inheritance of tissue-specific methylation patterns are distinguished. DNMT3A and DNMT3B catalyze *de novo* DNA methylation, whereas DNMT1 mediates the maintenance of DNA methylation [9]. DNMT1 is the maintenance cytosine-5-methyltransferase and binds methyl groups to the hemimethylated DNA

during replication, while DNMT3A and DNMT3B exhibit *de novo* methyltransferases' activity and add methyl groups to CpG dinucleotides of unmethylated DNA to create new patterns of methylation [12]. DNA methylation by Dnmt proteins in the promoter regions is associated with gene silencing, thus linking DNA methylation to chromatin inaccessibility and gene suppression [13]. Evidences from other recent studies have also suggests and clarified the roles of DNA methylation in gene bodies and intergenic regions in enhancing gene expression [14]. Accurate global understanding of the DNA methylation process is crucial in the context of a potential cultivation of myocardial progenitor cells because cell differentiation status is defined by the gene expression profile, which is coordinately controlled by epigenetic mechanisms.

Since cardiomyocytes were commonly considered to be terminally differentiated and do not proliferate significantly under physiological conditions [1], epigenetic mechanisms—among them DNA methylation—seemed of little importance for heart disease. However, an increasing number of studies in recent years indicate, that in cardiomyocytes, dynamic CpG methylation is not only predominantly confined to postnatal development, but also occurred in experimental heart failure [15]. Gilsbach et al. employed cardiomyocytes of neonatal, adult healthy and adult failing hearts and demonstrated large genomic regions that are differentially methylated during cardiomyocyte development and maturation [15]. These findings unquestionably indicated, in contrast to the established views, that DNA methylation is a highly dynamic process during postnatal growth of cardiomyocytes and their adaptation to pathological stress in a process tightly linked to gene regulation and activity. Other investigators [16], also found evidences supporting dynamic nature of DNA methylation. In hearts from patients with end-stage cardiomyopathy undergoing heart transplantation, intra-genic CpG islands displayed higher methylation levels compared to control [16]. In our study, using RT-qPCR approach, we found increased expression of DNMT1 and DNMT3A mRNA levels during long-term *in vitro* cell culture. Moreover, transcript expression levels showed a growing character during the cultivation period. These results, in line with described above studies, confirmed importance of epigenetic mechanisms in cardiomyocytes activity.

Although, cardiomyocytes show low rates of DNA synthesis postnatally [3], and DNMT1, as the maintenance methyltransferase, does not emerge as a key player of the observed hypermethylation, some research found that DNMT1-mediated DNA methylation is increased in right ventricular fibroblasts (RVfib) in pulmonary arterial hypertension (PAH) [17]. These studies demonstrated, that DNMT1 via methylation activate HIF-1 α (hypoxia-inducible

factor) and cause a proliferative, fibrogenic RVfib phenotype. Furthermore, HIF-1 α upregulates PDK (pyruvate dehydrogenase kinase) expression and increases fibrogenic cytokines, like TGF- β 1 (transforming growth factor-beta-1) and CTGF (connective tissue growth factor). This pathway additionally promotes collagen production and right ventricular fibrosis in PAH [17]. In the present study, we also found increasing DNMT1 expression during *in vitro* cultivation. According to the research described above, such a potentially increasing DNMT1 activity may correlate with the fibrosis in a cultured cell mix, which would undoubtedly have a negative effect on downstream applications employed these cells.

The assumption that DNMT3A and DNMT3B are responsible for *de novo* CpG methylation in the absence of DNA replication makes that, *de novo* CpG methylation in cardiomyocytes is likely caused by DNMT3A and/or DNMT3B. The most numerous studies attempt to characterize precisely the role of these two DNA methyltransferases in the myocardium. In the present study, we demonstrated markedly lower transcript levels of DNMT3B when comparing with DNMT3A expression levels. These results suggest that Dnmt3a seems to be the main enzyme which exhibits *de novo* methyltransferases' activity in cultured cell population. Nührenberg et al. employing mice with specific ablation of *Dnmt3a* and *Dnmt3b* (DKO) expression in cardiomyocytes, identified upregulation of 44 and downregulation of 9 genes in DKO as compared with control. Promoters of upregulated genes were largely unmethylated in DKO compared to control mice. Nevertheless, both mice without (sham) and with left ventricular pressure overload induced by transverse aortic constriction (TAC), showed similar changes with substantial overlap of regulated genes [18]. The author concludes that *de novo* DNA methylation in cardiomyocytes is dispensable for adaptive mechanisms after chronic cardiac pressure overload [18]. Other investigators [19], employing similar approach, used DNMT3A knockout human induced pluripotent stem cell-derived cardiomyocytes, and performed wide molecular, histological, and ultrastructural analyses. Of the numerous results obtained, the authors also observed correlation between DNA methylation and HIF-1 α functionality, rendering knockout-engineered heart tissue sensitive to metabolic stress such as serum withdrawal and restrictive feeding [19]. Naito et al. on skeletal muscles satellite cells model with use Dnmt3a-conditional knockout (cKO) mice also confirmed, that Dnmt3a is found to maintain muscle homeostasis by epigenetically regulating the proliferation of satellite cells (SCs) [20]. Research using the satellite cell model seems to be particularly important in the context of the cardiac muscle, and potential population of cardiac progenitor cells (CPCs), which would play a similar role in the heart muscle to that of satellite cells in skeletal muscle.

Conclusions

The results of transcriptomic studies using RT-qPCR presented in this manuscript indicate an active process of methylation in cardiac muscle cells cultured under *in vitro* conditions. We showed up-regulation of transcript levels for main DNA methyltransferases during cultivation. However, to clarify final effects of increased DNMT1 and DNMT3A mRNA levels, other studies are needed. It will be necessary to indicate the place where the covalent attachment of the methyl group occurs, in the promoter region or in gene bodies. Research with the use of DNMTs inhibitors would also significantly increase the knowledge about the impact of the methylation process on the phenotype and genotype of cultured cells.

Ethical approval

As the research material is usually disposed of after slaughter, being a remnant by-product, no ethical committee approval is needed for this study.

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Conflict of interest statement

The authors declare they have no conflict of interest.

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