Abstract

Duchenne muscular dystrophy (DMD) is an X-linked genetic disorder caused by mutations in the *DMD* gene encoding dystrophin, a protein that plays an important role in maintaining the integrity of the muscle cell membrane. The lack of this protein leads thus to progressive muscle weakness; however, in the later stages of the disease it is also associated with respiratory failure and cardiomyopathy, which is the main cause of death in DMD patients. The mechanisms of its development have not been fully understood yet, therefore, the aim of this study was to investigate the molecular background of DMD-associated cardiomyopathy.

In this study, human induced pluripotent stem cells (hiPSC)-derived cardiomyocytes (hiPSC-CM) were utilized as a research model. For this purpose, blood samples were taken from two healthy (unaffected) donors and one patient with DMD, and the peripheral blood mononuclear cells were isolated and reprogrammed using Sendai vectors. Subsequently, three pairs of isogenic hiPSC lines were generated by introducing a mutation in the *DMD* gene (deletion of exon 50) in the control (unaffected) hiPSC obtained from two healthy donors and repair of the mutation in the *DMD* gene in the hiPSC obtained from the DMD patient using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) (CRISPR/Cas9) gene editing system. All hiPSC lines were subjected to detailed characteristics confirming their pluripotency and optimization of cardiac differentiation conditions.

The first objective of the study was to perform the global transcriptomic and proteomic analysis of DMD hiPSC-CM in comparison to their control counterparts. These analyzes revealed several altered molecular pathways and processes within the cytoskeleton, cell membrane, and extracellular matrix, which is a direct effect of dystrophin deficiency. Interestingly, the results demonstrated changes in the gene expression and/or protein level of the factors involved in calcium metabolism, oxidative stress, and cardiac dysfunction. Moreover, a down-regulation of mitoNEET, the protein encoded by the CDGSH iron-sulfur domain-containing protein 1 (*CISD1*) gene, was observed. Due to the fact that the functions of this protein in DMD cardiomyocytes have never been described in the literature, therefore, it was subjected to more in-depth investigation. The protein is involved in regulation of iron homeostasis in cells; therefore, the levels of mitochondrial and cytosolic labile iron pool were measured, which revealed an increased labile iron concentration both in the mitochondria

and cytosol of dystrophin-deficient cardiomyocytes. Correspondingly, the decreased level of ferroportin and the increased level of ferritin in DMD hiPSC-CM are other characteristics of iron overload. One of the main consequences of iron overload is increased oxidative stress and excessive production of reactive oxygen species (ROS). In our studies, ROS production tends to increase in DMD hiPSC-CM and the cells have markedly elevated levels of Nrf-2 and NAD(P)H dehydrogenase [quinone] 1, proteins responsible for maintaining cellular redox balance. Interestingly, we demonstrated that the level of ROS production may be a direct effect of the iron content in the cells, as stimulation with an iron chelator, deferoxamine, tended to decrease the level of ROS, while stimulation with iron increased its level in DMD hiPSC-CM. The ultrastructure of dystrophic cardiomyocytes imaged using transmission electron microscopy (TEM) showed areas of degenerated mitochondria with damaged mitochondrial cristae structure.

In the next step, we investigated the functional effect of calcium metabolism disturbances in DMD hiPSC-CM by measuring calcium oscillations. Several pathological characteristics of these oscillations were described, such as the increased frequency of prolonged plateau occurrence, the reduced frequency of the oscillations, and the higher frequency of longer intervals between oscillations. Disturbances in calcium handling are also probably related to the observed increase in DMD hiPSC-CM stiffness. On the other hand, patch-clamp measurements of single DMD cardiomyocytes showed increased afterhyperpolarizations (AHP) values and decreased action potential duration at 20% repolarization in comparison to control cells.

The next objective of the study was to evaluate the role of utrophin in DMD-related cardiomyopathy. Utrophin is a protein that shares a high structural and functional similarity with dystrophin, and it was hypothesized that it can at least partially compensate for the lack of dystrophin. Many studies have been performed using the so-called microutrophin, the delivery of which to cells was associated with a therapeutic effect in *mdx* mice; however, its role in heart and cardiomyocytes has never been understood. In the present study, the effects of both utrophin knockout and utrophin expression activation in DMD hiPSC-CM were investigated.

Cardiomyocytes obtained from hiPSC differentiation have the immature phenotype and, as shown in this study, still express utrophin. Thus, all *in vitro* studies performed on the DMD hiPSC-CM model may be thus distorted by the presence of utrophin. In this study, we

evaluated whether the lack of both dystrophin and utrophin worsens the phenotype of hiPSC-CM in comparison to cells deficient in DMD only. Interestingly, we observed a significant deterioration in calcium handling, while there were no major signs of aggravation in action potential parameters and cell stiffness. This discrepancy may be explained by the location of utrophin in cardiomyocytes, which, apart from the sarcolemma, was also found in intercalated discs, as previously shown in murine heart. Thus, it may affect signal transmission, resulting in calcium handling abnormalities, but it does not affect the electrophysiological parameters of the cell itself.

Our next purpose was to evaluate whether activation of utrophin expression in DMD hiPSC-CM can rescue their pathological phenotype. To activate utrophin expression, CRISPR/deadCas9 (dCas9)-mediated transcriptional activation system based on the catalytically inactive Cas9 and VP64 activation domain was applied using adeno-associated virus (AAV) vectors. DMD hiPSC-CM with activated utrophin expression were subjected to patch-clamp measurements which demonstrated restored AHP values (increased in DMD hiPSC-CM) at the level of control cells.

In conclusion, hiPSC-CM provide a powerful methodology for studies of DMD-associated cardiomyopathy. The global transcriptomic and proteomic analyzes of dystrophic cardiomyocytes revealed changes in several pathways important for proper heart function (calcium metabolism, oxidative stress). Obtained data highlighted also severe cytosolic and mitochondrial iron overload in DMD hiPSC-CM. This observation has never been described so far, while it may provide novel therapeutic possibilities related to iron chelators, for instance. The lack of utrophin in DMD hiPSC-CM aggravates the observed calcium handling pathology, while it does not influence the action potential of single cardiomyocytes, while activation of utrophin expression rescues the increase in AHP in DMD hiPSC-CM.