

**Miniature CRISPR/Cas nucleases for upregulation of utrophin expression:  
a novel approach for mutation-independent therapy of Duchenne muscular dystrophy**

Duchenne muscular dystrophy (DMD) is an incurable disease that affects approximately 1 in 5,000 boys, as this is a sex-linked disorder. The first symptoms of the disease appear around the age of 2-5, causing the loss of ambulation in early teenage years and inevitably leading to death, currently most often in the third decade of life. DMD is caused by mutations in the dystrophin gene (DMD), located on the X chromosome. It is the largest human gene, and the number of known mutations, most often leading to a complete lack of this protein, is currently over 7,000.

The lack of dystrophin, necessary for the proper functioning of skeletal muscles, respiratory muscles (diaphragm), and the heart, as well as in the nervous system, leads primarily to damage to the limb muscles. However, the main cause of premature death in DMD patients is the developing heart muscle failure caused by damage to the function of heart cells, cardiomyocytes, leading to the development of cardiomyopathy.

So far, there has been no effective treatment for DMD. In the initial period, the progression of the disease can be stopped by using corticosteroids. Recently, genetic therapies that restore the expression of a shortened form of dystrophin have also been registered conditionally, but the effectiveness of these therapies has not yet been proven. However, apart from many technical problems, therapies restoring dystrophin expression are fraught with other, additional, important problems. Therapies based on antisense oligonucleotides that restore protein production can only be used in patients with a specific type of mutation. In addition, there is also the risk of an immune reaction to dystrophin. Therefore, one of the methods considered for DMD therapy is to restore the expression of utrophin, the equivalent of dystrophin present in muscles and cardiomyocytes only in the fetal period. However, the methods of utrophin induction tested so far, including pharmacological ones, have been proven to be ineffective.

In the submitted project, the Polish team from the Department of Medical Biotechnology of the Faculty of Biochemistry, Biophysics and Biotechnology of Jagiellonian University and the Lithuanian team from the European Molecular Biology Laboratory (EMBL) of the University of Vilnius propose the use of an innovative strategy for restoring utrophin expression by using the gene editing mechanism known as CRISPR/Cas. The proposed approach is comprehensive and uses the previous experience of both teams. Thanks to the use of unique, miniature forms of Cas proteins, recently discovered by the Lithuanian team, it will be possible to effectively introduce them into heart cells using effective AAV vectors constructed by the Polish team. The research will be conducted on human cardiomyocytes obtained by differentiating induced pluripotent stem cells from DMD patients obtained by the Polish team. As part of the study, both teams will test several ways to increase utrophin expression in cardiomyocytes from DMD patients. An original solution will be to test the effectiveness of the developed tools in three-dimensional systems composed of cardiomyocytes, endothelial cells, and fibroblasts, i.e. the main types of cells found in the heart.

The project is aimed at finding the most optimal strategy for inducing utrophin expression. The proposed solutions aim to use the editing of various areas of the utrophin gene, and through the simultaneous use of several approaches, find the most effective method, which should result in improving the functions of contractile cardiomyocytes and other properties of these cells. The implementation of the project in the proposed form is possible thanks to the combination of unique skills and experience of both teams, creating an opportunity to obtain the most optimal results.