M U N I

COMMENTARY TO HABILITATION THESIS[1]

Characteristics of the Investigated Matter

The habilitation thesis investigates the cellular consequences of dystrophin deficiency, with particular emphasis on cardiac pathology associated with Duchenne and Becker muscular dystrophies. Although dystrophin-associated cardiomyopathy is traditionally investigated primarily in the context of differentiated cardiomyocytes, the studies presented in this thesis address a broader question: whether dystrophin deficiency also affects stem and progenitor cell populations and whether abnormalities in these populations may be relevant to the development and progression of cardiac disease.

The research combines two complementary experimental approaches. The first employs human pluripotent stem cells, including patient-specific induced pluripotent stem cells, to investigate the functional consequences of dystrophin deficiency in differentiated cardiac models and cellular abnormalities detectable before cardiac differentiation. The second examines resident cardiac cell populations with stem/progenitor-like properties in human Becker muscular dystrophy myocardium and in the dystrophin-deficient mdx mouse model.

Together, these approaches provide a framework for studying dystrophin-associated cardiac pathology at different levels of cellular organization, from pluripotent stem cells and their differentiated cardiac derivatives to resident cell populations within the diseased heart. The central perspective of the thesis is that understanding dystrophin-associated cardiomyopathy requires consideration not only of cardiomyocyte dysfunction but also of abnormalities affecting other cellular populations potentially involved in cardiac development, maintenance and disease progression.

Objectives of the Work

The principal objective of the habilitation thesis is to investigate the cellular consequences of dystrophin deficiency in the context of dystrophin-associated cardiac pathology, encompassing both the functional impairment of differentiated cardiomyocytes and abnormalities affecting pluripotent stem cells and resident cardiac stem/progenitor-like populations. The thesis aims to integrate these complementary experimental perspectives to provide a broader understanding of the cellular processes associated with the development and progression of dystrophin-associated cardiomyopathy.

The specific objectives are to:

1. Establish and characterize human experimental models of dystrophin-associated cardiac disease, including patient-specific induced pluripotent stem-cell lines, and develop approaches for the quantitative assessment of functional abnormalities in their differentiated cardiac derivatives.

2. Investigate the consequences of dystrophin deficiency at the pluripotent stem-cell stage, particularly alterations in redox homeostasis, nitric oxide synthase activity and genomic integrity, and examine the role of reactive oxygen species in signalling pathways involved in pluripotent stem-cell regulation.

[1] The commentary must correspond to standard expectations in the field and must include a brief characteristic of the investigated matter, objectives of the work, employed methodologies, obtained results and, in case of co-authored works, a passage characterising the applicant's contribution in terms of both quality and content.

3. Characterize resident cardiac stem/progenitor-like cell populations in dystrophin-associated cardiac pathology, focusing on their abundance, cellular properties and the plasticity of affected myocardial tissue in human Becker muscular dystrophy.

4. Examine changes in resident cardiac progenitor-like populations during disease progression, using the dystrophin-deficient mdx mouse model to investigate age-dependent alterations in their abundance and distribution and to assess associated cellular abnormalities, including DNA damage.

Employed Methodologies

The research presented in this habilitation thesis employed complementary experimental models and analytical approaches to investigate the cellular consequences of dystrophin deficiency. The methodological strategy progressed from the establishment of human disease models and their functional characterization to the investigation of stem/progenitor cell populations and the molecular processes potentially underlying their abnormalities.

1. Establishment of experimental models

Patient-specific human induced pluripotent stem-cell lines were generated from individuals with Duchenne muscular dystrophy and characterized for pluripotency, genomic stability and retention of the disease-associated mutations. These models were complemented by dystrophin-deficient human embryonic stem cells and their isogenic wild-type controls. Directed cardiac differentiation was used to generate cardiomyocytes and three-dimensional cardiac aggregates suitable for functional and molecular investigations. To extend the research beyond in vitro models, explanted human myocardium from a patient with Becker muscular dystrophy and hearts from dystrophin-deficient mdx mice were also investigated.

2. Functional validation of stem-cell-derived cardiac models

The ability of human pluripotent stem-cell-derived cardiac models to reproduce disease-relevant functional abnormalities was assessed using complementary electrophysiological, biomechanical and calcium-imaging approaches. Microelectrode-array recordings and patch-clamp electrophysiology were used to characterize electrical activity, while atomic force microscopy enabled quantitative analysis of contractile behaviour. Combined AFM and microelectrode-array measurements permitted synchronized assessment of electrical and mechanical events in three-dimensional cardiac aggregates. Intracellular calcium dynamics and responses to pharmacological stimulation were examined to characterize excitation–contraction coupling and functional responsiveness.

3. Investigation of stem/progenitor cell abnormalities

Abnormalities at the pluripotent stem-cell stage were investigated using cellular and molecular assays assessing redox homeostasis, DNA damage and genomic integrity. The investigation was extended to resident cardiac c-kit^{+}/CD45^{-} populations isolated from human Becker muscular dystrophy myocardium and mdx mouse hearts. Cell abundance, spatial distribution, survival and ex vivo properties were assessed using tissue analysis, cell isolation and culture-based approaches. Age-dependent changes in the resident population were examined in the context of myocardial pathology and cardiac function, including histological analyses and echocardiography.

4. Investigation of molecular mechanisms

Potential molecular mechanisms were investigated using complementary biochemical, molecular and pharmacological approaches. The contribution of nitric oxide synthase activity

and reactive oxygen species to DNA damage was examined through measurements of oxidative stress and genomic damage, pharmacological inhibition, ROS scavenging and targeted knockdown of NOS isoforms. In parallel, the role of redox homeostasis in pluripotent stem-cell signalling was investigated through experimental modulation of intracellular ROS and analysis of PTEN and PI3K/AKT pathway activity. DNA-damage and ROS assays were also applied to isolated resident cardiac progenitor-like cells to assess whether related cellular abnormalities could be detected across the experimental systems.

Obtained Results

1. Establishment of Experimental Models

Two patient-specific induced pluripotent stem-cell lines carrying distinct deletions in the *DMD* gene were successfully established and characterized. Both lines retained their disease-associated mutations and fulfilled the criteria for pluripotency, including expression of pluripotency markers and the capacity to differentiate into derivatives of all three germ layers. Their genomic integrity and identity were also verified, providing well-characterized human cellular models of Duchenne muscular dystrophy.

Together with an isogenic system comprising dystrophin-deficient human embryonic stem cells and their genetically matched control, these patient-specific lines provided complementary experimental platforms for investigating the consequences of dystrophin deficiency. Their differentiation into cardiac cells and three-dimensional cardiac aggregates enabled subsequent studies of disease-associated functional abnormalities, while the undifferentiated pluripotent cells allowed investigation of cellular alterations preceding cardiac differentiation.

2. Cardiomyocyte Dysfunction

Cardiac cells derived from dystrophin-deficient human pluripotent stem cells exhibited functional abnormalities consistent with key aspects of dystrophin-associated cardiac pathology. Across patient-specific and isogenic experimental models, dystrophin deficiency was associated with alterations in spontaneous electrical and mechanical activity, impaired intracellular calcium handling and reduced responsiveness to β-adrenergic stimulation. A reduced proportion of spontaneously contracting cardiac aggregates further indicated compromised functional development or maintenance of the differentiated cardiac phenotype.

The studies also demonstrated the feasibility of quantitatively assessing the electromechanical behaviour of three-dimensional stem-cell-derived cardiac aggregates by combining atomic force microscopy with microelectrode-array recordings. This approach enabled simultaneous characterization of contraction dynamics and extracellular electrical activity, providing a complementary functional platform for investigating disease-associated phenotypes and pharmacological responses.

Together, these findings established that human stem-cell-derived cardiac models can reproduce multiple functional consequences of dystrophin deficiency. They also provided the differentiated-cell reference point for the subsequent investigation of abnormalities detectable before cardiac differentiation and in resident cardiac stem/progenitor-like populations.

3. Stem/Progenitor Cell Abnormalities

Abnormalities associated with dystrophin deficiency were identified not only in differentiated cardiac cells but also at the pluripotent stem-cell stage. Dystrophin-deficient human pluripotent stem cells exhibited elevated intracellular reactive oxygen species and increased DNA damage, demonstrating that cellular disturbances can be detected before cardiac differentiation and independently of the contractile dysfunction characteristic of dystrophic muscle.

The investigation of resident cardiac stem/progenitor-like populations revealed further abnormalities in dystrophin-associated heart disease. In explanted myocardium from a patient with end-stage Becker muscular dystrophy, the c-kit^{+}/CD45^{-} cell population was markedly

reduced compared with non-dystrophic donor myocardium. Cells isolated from the affected heart exhibited impaired ex vivo survival, while myocardial explant cultures showed reduced tissue plasticity. These findings indicated that advanced dystrophin-associated cardiac pathology involves alterations extending beyond differentiated cardiomyocytes.
Analysis of dystrophin-deficient *mdx* mice revealed that changes in the resident c-kit^{+}/CD45^{-} population were age-dependent rather than uniformly progressive. An increased proportion of these cells was observed in young *mdx* hearts, followed by a marked decline at later ages, accompanied by changes in their spatial distribution. Cells isolated from *mdx* hearts also exhibited increased DNA damage, although a corresponding significant increase in intracellular reactive oxygen species was not detected. These cellular changes occurred in the context of progressive myocardial fibrosis and impaired cardiac mechanics.
Together, these findings demonstrate that dystrophin-associated pathology involves abnormalities in distinct stem and progenitor-like cell populations. Although the studies do not establish a direct causal relationship between these abnormalities and cardiomyopathy progression, they identify additional cellular dimensions of the disease that warrant consideration alongside cardiomyocyte dysfunction.

4. Molecular Mechanisms and Their Limitations

Investigation of the molecular consequences of dystrophin deficiency in human pluripotent stem cells identified dysregulated nitric oxide synthase (NOS) activity as a major contributor to oxidative stress and genomic damage. Pharmacological NOS inhibition and targeted knockdown of NOS isoforms reduced DNA damage, while NOS inhibition and reactive oxygen species scavenging attenuated the associated redox and DNA-damage phenotypes. These findings provided experimental evidence that NOS-dependent ROS production contributes to genomic instability in dystrophin-deficient pluripotent stem cells.
Complementary experiments demonstrated that intracellular ROS also participate in the regulation of signalling pathways governing human pluripotent stem-cell behaviour. In particular, redox-dependent modulation of PTEN activity was linked to changes in PI3K/AKT signalling, establishing a broader role for ROS in pluripotent stem-cell regulation beyond their contribution to oxidative damage. However, these signalling experiments were not performed specifically in dystrophin-deficient cells and therefore do not establish PTEN/PI3K/AKT dysregulation as a mechanism of dystrophin-associated pathology.
The increased DNA damage detected in resident c-kit^{+}/CD45^{-} cells from *mdx* hearts suggests that genomic integrity may represent a relevant point of comparison between the investigated cell populations. However, whether a common molecular mechanism underlies DNA damage in dystrophin-deficient pluripotent stem cells and resident cardiac progenitor-like cells remains to be established, particularly as increased ROS levels were not demonstrated in the latter population under the experimental conditions employed. Thus, the studies identify mechanistically defined abnormalities in dystrophin-deficient pluripotent cells and potentially related, but not yet mechanistically linked, alterations in resident cardiac stem/progenitor-like populations.

Applicant's Contribution

My principal contribution to the research presented in this habilitation thesis was the scientific development, direction and coordination of a research programme investigating the cellular consequences of dystrophin deficiency. Building on the initial proposal by Albano Meli to investigate Duchenne muscular dystrophy using human pluripotent stem-cell models, I developed and directed the establishment of patient-specific DMD hiPSC lines and their subsequent application to cardiac disease modelling. Drawing on my previous research into DNA damage and genomic stability, I conceived the hypothesis that dystrophin deficiency might compromise genome integrity already at the pluripotent stem-cell stage and directed the investigation of NOS-dependent oxidative stress and DNA damage. Together with Petr Fojtík, I also developed a complementary research direction addressing redox-dependent regulation

of signalling pathways in human pluripotent stem cells. I subsequently extended the programme to resident cardiac stem/progenitor-like populations, formulating and directing investigations of their abnormalities in human Becker muscular dystrophy myocardium and during disease progression in the *mdx* mouse model.

Most of the stem-cell and cellular experimental work was conducted in my laboratory by students and researchers under my supervision. My contribution included the design of experimental strategies, coordination and supervision of laboratory activities, personal participation in selected experiments, interpretation and integration of results, and substantial involvement in manuscript preparation. I supervised the development and characterization of human cellular models, investigations of their cardiac phenotypes, and studies of redox homeostasis, genomic integrity and resident cardiac cell populations. My role was particularly focused on connecting the findings from different experimental systems into a coherent research programme addressing cellular consequences of dystrophin deficiency beyond differentiated cardiomyocytes.

Several studies relied on essential contributions from collaborating researchers and laboratories. Martin Pešl coordinated access to patient material and clinical collaborations, including the explanted Becker muscular dystrophy myocardium. The combined AFM–MEA biosensing system was developed by Roberto Raiteri's group, with electromechanical measurements performed in Jan Přibyl's laboratory. Albano Meli contributed electrophysiological expertise and supervised experimental work conducted in Alain Lacampagne's laboratory in France, including a substantial part of the *mdx* mouse study performed there by my PhD student Šárka Jelínková. My responsibility within these collaborations was to develop and coordinate the biological research strategy, supervise the work conducted within my group, integrate the complementary experimental findings and contribute to their scientific interpretation and publication. The quantitative extent of my involvement in individual studies is specified in the contribution tables accompanying the seven publications.

[1] JELINKOVA, Sarka, Lenka MARKOVA, Martin PESL, Iveta VALÁŠKOVA, Eva MAKATUROVÁ, Lenka JURIKOVA, Petr VONDRACEK, Alain LACAMPAGNE, Petr DVORAK, Albano C. MELI and **Vladimir ROTREKL**. Generation of two Duchenne muscular dystrophy patient-specific induced pluripotent stem cell lines DMD02 and DMD03 (MUNIi001-A and MUNIi003-A). *Stem Cell Research* [online]. 2019, **40**(Article 101562). ISSN 1873-5061. Available at: doi:10.1016/j.scr.2019.101562

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
20	100	50	80

[2] CALUORI, Guido, Jan PRIBYL, Martin PESL, Sarka JELINKOVA, **Vladimir ROTREKL**, Petr SKLADAL and Roberto RAITERI. Non-invasive electromechanical cell-based biosensors for improved investigation of 3D cardiac models. *Biosensors and Bioelectronics* [online]. 2019, **124–125**, 129–135. ISSN 0956-5663. Available at: doi:10.1016/j.bios.2018.10.021

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
20	50	50	50

[3] JELINKOVA, Sarka, Aleksandra VILOTIC, Jan PRIBYL, Franck AIMOND, Anton SALYKIN, Ivana ACIMOVIC, Martin PESL, Guido CALUORI, Simon KLIMOVIC, Tomas URBAN, Hana DOBROVOLNA, Vladimir SOSKA, Petr SKLADAL, Alain LACAMPAGNE, Petr DVORAK,

Albano C. MELI and **Vladimir ROTREKL**. DMD Pluripotent Stem Cell Derived Cardiac Cells Recapitulate in vitro Human Cardiac Pathophysiology. *Frontiers in Bioengineering and Biotechnology* [online]. 2020, **8**(Article 535). ISSN 2296-4185. Available at: doi:10.3389/fbioe.2020.00535

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
20	100	50	80

[4] JELINKOVA, Sarka, Petr FOJTIK, Aneta KOHUTOVA, Aleksandra VILOTIC, Lenka MARKOVÁ, Martin PESL, Tereza JURAKOVA, Miriama KRUTA, Jan VRBSKY, Renata GAILLYOVA, Iveta VALÁŠKOVÁ, Ivan FRÁK, Alain LACAMPAGNE, Giancarlo FORTE, Petr DVORAK, Albano C. MELI and **Vladimir ROTREKL**. Dystrophin deficiency leads to genomic instability in human pluripotent stem cells via NO synthase-induced oxidative stress. *Cells* [online]. 2019, **8**(1, Article 53). ISSN 2073-4409. Available at: doi:10.3390/cells8010053

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
20	100	50	100

[5] FOJTÍK, Petr, Deborah BECKEROVÁ, Katerina HOLOMKOVÁ, Martin ŠENFLUK and **Vladimir ROTREKL**. Both Hypoxia-Inducible Factor 1 and MAPK Signaling Pathway Attenuate PI3K/AKT via Suppression of Reactive Oxygen Species in Human Pluripotent Stem Cells. *Frontiers in Cell and Developmental Biology* [online]. 2021, **8**(Article 607444). ISSN 2296-634X. Available at: doi:10.3389/fcell.2020.607444

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
20	100	50	80

[6] PESL, Martin, Sarka JELINKOVA, Guido CALUORI, Maria HOLICKA, Jan KREJCI, Petr NEMEC, Aneta KOHUTOVA, Vita ZAMPACHOVA, Petr DVORAK and **Vladimir ROTREKL**. Cardiovascular progenitor cells and tissue plasticity are reduced in a myocardium affected by Becker muscular dystrophy. *Orphanet Journal of Rare Diseases* [online]. 2020, **15**(1, Article 65). ISSN 1750-1172. Available at: doi:10.1186/s13023-019-1257-4

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
20	80	30	80

[7] JELINKOVA, Sarka, Yvonne SLEIMAN, Petr FOJTÍK, Franck AIMOND, Amanda FINAN, Gerald HUGON, Valerie SCHEUERMANN, Deborah BECKEROVÁ, Olivier CAZORLA, Marie VINCENTI, Pascal AMEDRO, Sylvain RICHARD, Josef JAROS, Petr DVORAK, Alain LACAMPAGNE, Gilles CARNAC, **Vladimir ROTREKL** and Albano C. MELI. Dystrophin deficiency causes progressive depletion of cardiovascular progenitor cells in the heart. *International Journal of Molecular Sciences* [online]. 2021, **22**(9, Article 5025). ISSN 1661-6596. Available at: doi:10.3390/ijms22095025

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
20	60	30	80