ACKNOWLEDGEMENT

This master project was carried out at Cardiac Exercise Research Group (CERG) at the Department of Circulation and Medical Imaging at the Norwegian University of Science and Technology (NTNU), Trondheim, in the period of July 2014 to June 2015, leading to a master degree in Molecular Medicine.

I would like to express my gratitude to my supervisor Gustavo Jose Justo da Silva for taking me in as a master student in CERG and for all the help he has given me through the year. I learned a lot from him in including molecular and biological lab techniques and methodology of research. His attitude to research work encouraged me a lot when I met obstacle.

I also thank Jose Bianco N. Moreira and Natale Pinheiro Lage Rolim who supervised me on in the lab and help me at any time without hesitate.

Thanks Christiano Robles Rodrigues Alves who made me available to us the cancer cachexia models Thanks for Oddgeir Lingaas Holmen and Anja Bye who gave access to the HUNT-MI and HUNT Fitness data, respectively.

Thanks to Ragnhild Røsbjørgen, Karin Solvang-Garten and Toril Rolfseng for being always helpful in the lab. Thanks everyone in CERG gave me so good experience in the passed year.

Thanks to Marit Barstad who always help me when I need to adjust the study plan.

Thanks for my family. I could not complete this master project without support from all of you.

Trondheim, May 2015

Jianhua Han

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ABSTRACT

Large-scale epidemiological studies demonstrate that aerobic capacity, measured as maximal oxygen uptake (VO_{2max}), is the single best predictor for future cardiovascular mortality in both healthy individuals and patients with cardiovascular diseases. However, limited progress has been observed in the identification of the genetic basis of aerobic capacity. Recently, our group was able to identify a new and uncharacterized aerobic capacity locus (rs540 on TMEM8A gene) on chromosome 16p13.3 in the general population from The Nord-Trøndelag Health (HUNT) Study. Therefore, this Master thesis aimed to dissect this locus and search for other candidate genes associated with aerobic capacity, as well as their clinical relevance. The reanalyzes of genetic variants located in the vicinity of chromosome 16p13.3 locus reveled the existence of another variant (rs3830160 on MRPL28 gene) significantly associated with VO_{2max}. We have also checked for association of several other MRPL28 variants in myocardial infarction (MI) patients from the HUNT MI Study but none of then reached significant levels. In experimental models, MRPL28 was found ubiquitously expressed in mice, especially in kidney, brain, testis and ovary. Transient transfection of HEK-293 cells with siRNA against MRPL28 was performed and proteins involved in the oxidative phosphorylation were evaluated, but we fail to knocked down MRPL28. Regarding pathological conditions, we did not find MRPL28 differently expressed in two different cancer cachexia models (Wistar rats injected with Walker 256 tumor and B16 melanoma). In contrast, MRPL28 protein levels were 1.78 fold increased in the left ventricle of HCR compared to LCR. Regarding MRPL28 post-transcriptional regulation, we found that hsa-miR-18a-5p, hsa-miR-130b-3p, hsa-miR-326 and hsa-miR-328-3p are predicted to target MRPL28. Taken together, we have described that MRPL28 is a promising candidate target for aerobic capacity. The identification and validation of new aerobic capacity genetic variants will bring forward a potential to establish original and novel diagnostic and therapeutic tools and strategies for cardiovascular disease (CVD) management, thus contributing to reduce the burden of cardiovascular and life-style related diseases.

ABBREVIATIONS

AHA	American heart association
AIT	Aerobic interval training
AVB	Atrioventricular block
CEU	Utah residents with ancestry from northern and western Europe
CHB	Han Chinese in Beijing
CME	Continuous moderate exercise
COPD	Chronic obstructive pulmonary disease
CVD	Cardiovascular disease
DMEM	Dulbecco´s modified eagles medium
DAYLs	Deaths and disability-adjusted life years
ETC	Electoral transport chain
FBS	Fetal bovine serum
HCR	High-capacity runner rat
HUNT	The Nord-Trøndelag Health Study
JPT	Japanese in Tokyo
LCR	Low-capacity runner rat
miRNA	microRNA
MRPL28	Mitochondrial ribosomal protein L28
OXPHOS	Oxidative phosphorylation
SNPs	Single-nucleotide polymorphisms
siRNA	Small interfering RNA
VO_{2max}	Maximal oxygen uptake
WHO	World health organization
YRI	Yoruba in Ibadan

1. INTRODUCTION

1.1 CVD and epidemiology

Cardiovascular diseases (CVD) are the leading global cause of death and cause around 17.3 million deaths per year (Mozaffarian et al, 2015). It is estimated that this number will grow to more than 23.6 million in the next twenty years (Mozaffarian et al, 2015). Moreover, the World Health Organization (WHO) estimates that around 80 percent of those deaths take place in low- and middle-income countries (Valentin Fuster, 2010). About 2,150 Americans die each day from CVDs, one every 40 seconds (Mozaffarian et al, 2015). Nearly half of all African-American adults have some forms of cardiovascular disease, 48 percent of women and 46 percent of men (Mensah & Brown, 2007). Comparing to other highly prevalent diseases, CVDs claim more lives than all forms of cancer combined. The American Heart Association (AHA) estimates that about 85.6 million Americans are living with some forms of cardiovascular disease or the after-effects of stroke (Mensah & Brown, 2007). Direct and indirect costs of cardiovascular diseases and stroke are total more than $320.1 billion, including health expenditures and lost productivity (Mozaffarian et al, 2015).

1.2 Aerobic capacity and CVD

VO_{2max} (also known as maximal oxygen consumption or maximal oxygen uptake) is the maximum rate of oxygen consumption utilized during maximal or exhaustive exercise test, and it is measured as milliliters of oxygen per kilogram of body weight per minute (e.g., mL/(kg·min)). Aerobic capacity, which can be measured by VO_{2max}, is a stronger predictor for cardiovascular mortality both in health and CVDs patients than any other predictors (Kavanagh et al, 2002; Mora et al, 2007). More precisely, aerobic capacity has an independent protective effect against cardiovascular morbidity and all-cause mortality, both in the general population and in people with increased risk of cardiovascular diseases (Blair et al, 1989; Erikssen et al, 1998; Lin et al, 1999). Even small differences in VO_{2max} may predict substantial differences in cardiovascular health, as indicated by differences in risk factor levels (Carnethon et al, 2003; Petrella et al, 2005).

Our group has been focus on better describing the association between physical activity and related risk factor for the cardiovascular diseases on both health individuals and patients with CVDs. In the large population based study which 2368 man and 2263 women were included (www.ntnu.no/hunt/), we showed that the both women and man below the median VO_{2peak} (women <35.1 $mL \cdot kg^{-1} \cdot min^{-1}$; man <44.2 $mL \cdot kg^{-1} \cdot min^{-1}$) have several times increased the risk to have metabolic syndromes compared to those highest value of VO_{2peak} (women ≥40.8 $mL \cdot kg^{-1} \cdot min^{-1}$ and man ≥50.5 $mL \cdot kg^{-1} \cdot min^{-1}$) (Aspenes et al, 2011). Furthermore approximately 56% higher prevalence of metabolic syndrome in both genders is in consistent with 5 $ml \cdot kg^{-1} \cdot min^{-1}$ lower of VO_{2peak} (Aspenes et al, 2011).

1.3 Exercise training in prevention and treatment of CVD

Physical activity was estimated to play an important role in the improvement on health status and high level of life quality. Moreover, physical inactivity became more recently an independent risk factor diseases burden. According to the Global Burden Disease Study, physical inactivity (together with dietary risk factors) was responsible for the largest (10% of global DALYs) disease burden in passed ten years (Lim et al, 2012). Several clinical trials have shown that physical exercise is an effective intervention for some diseases, such as chronic obstructive pulmonary disease (COPD) (Garcia-Aymerich et al, 2006) and arthritis (Esser & Bailey, 2011). A meta-epidemiological study, designed to evaluate the effectiveness of physical exercise and drug intervention on the mortality outcomes of 339274 participants, showed that physical activity intervention was more efficient than drug intervention among patients with stroke (Naci & Ioannidis, 2013).

In the past years, our group has investigated the benefits of exercise training to patients under different CVDs. High-intensity interval training (HIT) was identified an important factor for reversing LV remodeling and improving aerobic capacity and quality of life in patients with post infarction heart failure by comparing different intensity of training interventions (moderate continuous training (CME) and aerobic interval training (AIT)) (Wisloff et al, 2007). In addition, we showed that physical activity would reverse the risk factor metabolic syndrome and improvement of VO_{2max} in patients with metabolic syndrome (Tjonna et al, 2008). In advance, the intensity of the exercise is an important factor to improve VO_{2max} and reversed metabolic means that AIT is more efficient than CME (Tjonna et al, 2008). Similar

benefits have been achieved when aerobic interval training was applied in patients with type 2 diabetes mellitus (Hollekim-Strand et al, 2014); heart failure patients after coronary artery bypass surgery (Moholdt et al, 2009) and overweight adolescents (Tjonna et al, 2009). More recently, we have started to enrolled participants for two others clinical trials in order to study the benefits of aerobic interval training in patients with diastolic heart failure (OptimEx-CLIN) (Suchy et al, 2014) and elderly people (Generation 100) (Stensvold et al, 2015).

1.4 Molecular and genetic basic for aerobic capacity

It is extremely important to precisely identify the genetic basis for VO_{2max}, which will provide foundation for studying mechanism and prevention of CVDs. To this purpose, a HERITAGE Familial Study performed VO_{2max} test in parents and their adult offspring from sedentary Caucasian decent families and revealed a maximal heritability of 51% for VO_{2max} adjusted by age, sex, body mass, fat-free mass, and fat mass (Bouchard et al, 1998). To identify human genomic regions associated to VO_{2max}, genomic scan was performed in twins and their families before and after a standardized endurance training program (Bouchard et al, 2000). The result of the study showed that chromosomes 4q, 8q, 11p, and 14q harbor several candidate genes for basal VO_{2max} and chromosomes 1p, 2p, 4q, 6p and 11q harbor genes that may be candidate genes for the trainability of VO_{2max} (Bouchard et al, 2000). Further, a GWAS study based on 324,611 single-nucleotide polymorphisms (SNPs) revealed that 39 SNPs association with the gains of VO_{2max} (Bouchard et al, 2011b). At the same time, the study shown that the more SNPs the subjects carried benefited more for improvement of VO_{2max} (Bouchard et al, 2011b).

To further investigate the fitness capacity and its gene regulation, HCR and LCR rat models were developed. HCR and LCR rat strains were produced by artificial selection and inbreeding of genetically heterogeneous N:NIH rat stocks based on the intrinsic running capacity on treadmill (Koch & Britton, 2001). By using of male and female rats from generations 14, 15, and 17 of HCR and LCR, we found that median lifespan of LCR animals is significantly shorter than HCR animals and Physical activity levels, VO_{2max} and lean body mass were sustained better in HCR than LCR during the process from adult to old status (Koch & Britton, 2001). In another study, the reduced fitness was suggested to mediate an impairment of mitochondrial function. This study found that mitochondria of HCR animals

were preferred to utilize BBCA and fat than LCR during the first period of exercise (Overmyer et al, 2015).

In addition, we revealed that 1540 were differentially expressed out of 28000 genes screened by microarray and gene ontology analysis from the LV of HCR and LCR rats (Bye et al, 2008a; Bye et al, 2008b). For example, acyl-coenzyme A dehydrogenase (short chain) associated with lipid metabolism and hemoglobin-chain complex (Hbb) associated with O_2 transported were found higher expression in HCR than LCR (Bye et al, 2008b). A transcript homology to LARS2 which encodes aminoacyl-tRNA synthesis and mitochondria aminoacyl-tRNA synthesis in human was found 65% higher in soleus muscle from the sedentary HCR than LCR (Bye et al, 2008a).

Moreover, several post-trasncriptional regulatory mechanisms could play a role in the aerobic capacity, including microRNAs (miRs). miRs, small endogenous single stranded noncoding which negatively regulate gene expression (Lau et al, 2001), were identified in circulations as biomarkers for CVDs, such as coronary artery disease (Han et al, 2015). miRs might involve in regulation process associated with VO_{2max}. Anja et al, in our group identified that miR-210, miR-21, and miR-222 association with low VO_{2max} in health population (Bye et al, 2013). Recently, metabolites were reported as biomarkers for VO_{2max}. Unsaturated fatty acids, phosphatidylcholine decreased while free choline and glucose increased in low VO_{2max} health individuals (Bye et al, 2012).

In another recent study conducted by our group, a new genetic variant (rs540) located in a transmembrane protein 8 (TMEM8A) was identified significantly associated with VO_{2max} in the HUNT3 Fitness cohort (unpublished data). Interestingly, rs540 variant is located in the TMEM8A 3'UTR and a single nucleotide substitution creates an alternative miRNA target site for hsa-let-7a-3p that affects TMEM8A gene expression. Moreover, we have shown that TMEM8A 1080T allele homozygous subjects had higher odds of having high waist circumference and obesity, and lower odds of having hypertension.

1.5 Mitochondria and mitochondrial diseases

Impairment of mitochondrial function was considered associated with reduction of VO_{2max} (Overmyer et al, 2015) and might link cardiovascular diseases and metabolic diseases as we

discussed before. Mitochondria are increasingly recognized as an important organelle and are involved several cellular processes especially in energy providing. Each human cell contains thousands of mitochondria and each mitochondria carries 2-10 copies of mitochondria DNA (mtDNA). MtDNA contains 37 genes: 13 polypeptides that involved in oxidative phosphorylation (OXPHOS), 2 ribosome RNAs (rRNA) and 22 transfer RNAs (tRNA). Mitochondria are responsible for generating most of the energy required by the cell and mitochondria supply nearly 90% of the heart's energy demand through oxidative phosphorylation (Marin-Garcia, 2013). The OXPHOS is the metabolic pathway in which mitochondria transfer the energy from oxidation of glucose and fat to ATP that can be used by all of physiology process in the body. The main reaction were occurred in electron transport chains (ETC) located in inner membrane of mitochondria comprised by five protein complex: NADH-coenzyme Q oxidoreductase (complex I); Succinate-Q oxidoreductase (complex II); flavoprotein-Q oxidoreductase Q-cytochrome c oxidoreductase (complex III); Cytochrome c oxidase (complex IV); ATP synthase (complex V).

Energy metabolism begins with the utilization of fat acid and glucose by β-oxidation and glycolysis. The product acetyl coenzyme A (CoA) from β-oxidation and glycolysis then was utilized in tricarboxylic acid (TCA) and generated high energy NADH and FADH2 productions. Electron from NADH and FADH2 was transferred to O_2 and generated a proton electrochemical gradient across the inner mitochondrial membrane. This gradient drives the complex IV (FO-F1 ATP synthase) producing ATP by phosphorylation of ADP.

Except generation of energy, mitochondria play important role on cellular stress responses, cell death, various signal transduction pathways, and intracellular Ca^{2+} homeostasis (Nunnari & Suomalainen, 2012). So that mitochondrial dysfunction is associated with various human disorders. Both the diseases caused by mutations in the mitochondrial and that mutations in the nuclear genes whose gene products conduct function in mitochondria are all called mitochondrial diseases (Nunnari & Suomalainen, 2012). Tissues that have high energy requirements such as heart, brain, muscle, kidney and the endocrine system more susceptible for mitochondrial pathology (Taylor & Turnbull, 2005). Dysfunction of cardiac mitochondrial metabolism has already been implicated in pathogenesis of ischemia, HF, dysrhythmias, hypertrophic cardiomyopaty and atherosclerosis (Marin-Garcia, 2013).

1.5.1 Mitochondria ribosome proteins (MRPs)

MRPs are a group of proteins encoded by nucleus genome named mitochondrial ribosome proteins gene that were part of original mitochondrial genome (O'Brien et al, 2005). Mitochondria derived from the Gram-negative bacterial progenitors 1.5-2 billion years ago (Dyall et al, 2004). An Gram-negative bacterial ancestor invaded in to an eukaryotic host cell, as an endosymbiont, changed into mitochondria (Dyall et al, 2004). Some of mitochondrial genome were transported into nucleus and resided in nucleus genome during the evolution process and these nucleus gene encoded proteins would be selectively transported back to mitochondria still conduct important role in mitochondria (Dyall et al, 2004). More than1000 proteins have been identified in mammalian mitochondria, but most of then are encoded by nuclear genes in mammal cells (Gray, 2012) and only 13 proteins are encoded by mtDNA in (Sylvester et al, 2004).

The chromosome location of most human MRPs gene has been determined and placed in mega base map (Kenmochi et al, 2001). All the MRPs are imported into mitochondria and construct mitochondrial ribosome (Kenmochi et al, 2001). The determination of the mammalian mitochondrial ribosomal structure (Greber et al, 2014; Koc et al, 2001) provides a foundation for the research on MRPs. Mitochondrial ribosome is composed of two subunits: the small subunit and large subunit. MRPL encoded proteins participate in composition of large subunit of mitochondrial ribosomal and MRPS encoded proteins participate in composition of small subunit of mitochondrial ribosomal (Sylvester et al, 2004). The small subunit of human mitochondrial ribosome contains a 12S rRNA and around 30 proteins and the large subunit contains 16S rRNA and approximately 48 proteins (Greber et al, 2014; Koc et al, 2001).

MRPL28 is located in the L1 stalk of the large subunit of mitochondrial ribosomal, which is the tRNA binding site (Greber et al, 2014) (Figure 1). Human MRPL28 was revealed longer than its prokaryotes homologous which might compensate for the truncated part of the mammalian mitochondrial 16s rRNA (Koc et al, 2001). MRPL28 closely connects with MRPL9, MRPL37 and MRPL47 (Greber et al, 2014). MRPL28 also extends toward the exit tunnel of the mitochondrial ribosome which is important site to guide the new generated proteins biogenesis (Gruschke & Ott, 2010). Phosphorylation status of MRPL28 and other protein in the L1 stalk could regulate interaction between the mitochondrial ribosomal and the

C-terminal tail of human Oxa1L which play a role in inserting new biogenesis proteins in mitochondrial inner membrane (Haque et al, 2010).

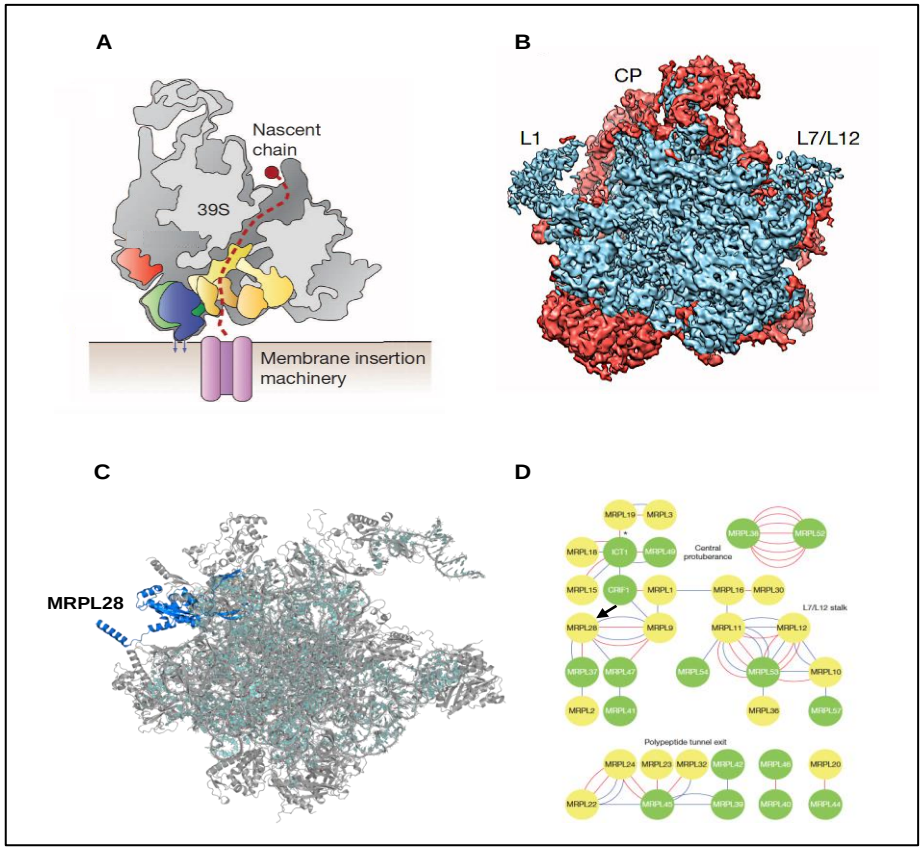

Figure 1. Architecture of the mammalian mitochondrial ribosome protein. A. Schematic illustration of the mammalian mitochondrial ribosome bound to the mitochondrial inner membrane; B. The structure of the 39S subunit segmented into conserved (blue) and mitochondrial ribosome-specific (red) density elements. CP, central protuberance; L1, L1 stalk; L7/L12, L7/L12 stalk base(Greber et al, 2014); C. MRPL28 assembly position in the 39S subunit is highlighted in blue; D. inter-protein crosslinks of 39S mitochondrial ribosomal subunit proteins (MRPL28 indicated with an arrow). Adapted from EMBL-EBI Protein Data Bank in Europe and Greber paper (Greber et al, 2014).

1.5.2 MRPs and CVDs

More recently, several clinical cases have been reported mutations in MRPs causing series clinical symptoms, especially neuropathology, hypertrophic cardiomyopathy, and metabolism

symptoms, such as lactose acidosis (Carroll et al, 2013; Galmiche et al, 2011; Miller et al, 2004; Saada et al, 2007; Smits et al, 2011). Patients carrying mutation in MRPs also present tissue specific deficiency in OXPHOS activity and in respiratory chain deficiency in complex I, III and IV in skeletal muscular or fibroblast (Carroll et al, 2013; Galmiche et al, 2011; Miller et al, 2004; Saada et al, 2007; Smits et al, 2011). The patients with MRPL44, MRPL16 mutations were identified with reduction of 16S rRNA and other protein assembled with large subunit of mitochondrial ribosomes (Carroll et al, 2013; Galmiche et al, 2011). Patients with mutation in MRPS22, MRPS16 were revealed with reduction of 12S rRNA (Miller et al, 2004; Saada et al, 2007; Smits et al, 2011). This suggested that large subunit and small subunit of mitochondrial ribosomal are relatively independent in maintain stability. Patients with mutations in MRPS22, MRPS16 all had dimorphism congenitally which might suggested mitochondrial small subunit had important function during the developing process(Carroll et al, 2013; Miller et al, 2004; Saada et al, 2007; Smits et al, 2011).

1.6 Study aims and hypothesis

Considering that MRPL28 were found associated with aerobic capacity and plays an important role in the mitochondrial DNA translation and function, we hypothesis that MRPL28 plays a role in different physiological (LCR/HCR) and pathological (MI, cancers) conditions. The potential mechanism of the association of MRPL28 with VO_{2max} might be involving the oxidative phosphorylation process.

Therefore, this Master project firstly aimed to describe tissue distribution of MRPL28 and to evaluate MRPL28 expression level in different physiological status and pathological status in both human and mouse, by accessing publicly available database and experimental models. To further evaluate the *in vitro* role of MRPL28, transient transfected HEK293 cells with siRNA. To further describe the post-transcriptional mechanisms to control MRPL28, we identify and validate miRNAs that target MRPL28.

2. METHODS

2.1 Population study

The Nord-Trøndelag Health Study (HUNT) study was carried out between the years of 2006 and 2008. This study is a sub-study in HUNT which was designed to measure VO_{2max} in health population was called the HUNT Fitness Study (Loe et al, 2013). 1472 non-smoking men were selected for genotyping from 4731 participants (Table 1). Exclusion criteria were diseases and habits could restrict their participants in the aerobic capacity measurement. No close relatives, first and second degrees relatives were included avoiding bias by genotyping. The study was approved by the regional committee for medical research ethics (REK nr: 4.2008.2792), the HUNT study, the Norwegian Data inspectorate, and by national Directorate of Health. The study was performed with Norwegian laws and the Helsinki declaration, and all participants signed a document of consent.

2.2 Databases mining

2.2.1 Single-SNP associative study in the HUNT fitness and HUNT MI SNPs database

Single-SNP logistic regression tests for association were performed only for the SNPs selected by the *in silico* approach, using PLINK package. The model was adjusted for age, physical activity levels (Kurtz score) and body mass index. SNPs and individuals with low genotype call rate (less than 95%) were excluded. SNPs with a minor allele frequency less than 5% were also excluded. Furthermore, SNPs that clearly deviates from the expected Hardy-Weinberg Equilibrium (HWE) were excluded ($p<10^{-7}$).

Table 1. Participant characteristics of the HUNT3 Fitness cohort.

	HUNT3 Fitness
Male sex, No. (%)	1472 (100)
Age (range), y	49.5±12.7 (19.6-84.4)
Anthropometric Data	
Height, cm	179.4±6.3 (161-200)
Weight, kg	85.9±10.9 (50.6-134.6)
Waist circumference, cm	94.9±8.8 (68-134)
Hip measurement, cm	102.9±5.9 (76-127)
Arterial blood pressure	
Systolic blood pressure, mm Hg	131.3±14.4 (95-186)
Diastolic blood pressure, mm Hg	76.0±10.5 (40-117)
Heart rate, bpm	66.5±11.1 (40-126)
Biochemical Data	
Total Cholesterol, mmol/L	5.51±0.99 (2.80-10.10)
High-density piloprotein, mmol/L	1.26±0.29 (0.60-2-60)
Glucose, mmol/L	5.55±1.56 (2.4-18.9)
Treadmill Data	
VO_{2max}, $mL \cdot kg^{-0.75} \cdot min^{-1}$	136.4±25.8 (65.15-222-06)
Physical activity index	
Kurtz Score	3.39±2.94 (0-15)

Data are presented as average ± standard deviation (minimum-maximum)

2.2.2 Evolutionary conservation regions

The genomic sequence in the vicinity of rs3830160 (chr16:419092) in MRPL28 (±1 Kb) from species representing different evolutionary clades (*Pan troglodytes, Bos taurus, Mus musculus, Canis familiaris, Monodelphis domestica, Gallus gallus, Xenopus laevis* and *Danio rerio*) were compared in order to detect conserved blocks shared by the genome of those species, using the ECR Browser (Ovcharenko et al, 2004).

2.2.3 Allele frequency population diversity

The datasets on genetic variation from both HapMap and 1000 Genomes projects were used in order to investigate the MRPL28 rs3803160 variant allele frequency in different populations: Utah residents with ancestry from northern and western Europe (CEU); African

Ancestry in South-Western United States (ASW), China (CHB, Han Chinese in Beijing), Japan (JPT, Japanese in Tokyo), Kenya (LWK, Luhya in Webuye), Nigeria (YRI, Yoruba in Ibadan) (Holm et al, 2010).

2.2.4 SNPexp

The SNPexp (version 1.2) web based tool was used to correlate the HapMap (Genome-wide SNP genotyping in 270 individuals from 6 populations) MRPL28 rs3803160 genotypes and MRPL28 gene expression levels (47294 transcripts) from GENEVAR (GENe Expression VARiation) database. This database was generated by the use of EBV-transformed lymphoblastoid cell lines from the same 270 HapMap individuals (Holm et al, 2010).

2.3 *In Vitro* experiments(Holm et al, 2010)

2.3.1 Cell culture

Human embryonic kidney cells (HEK-293 cells, CRL_1573) were obtained from ATCC (Rockville, MD) and grown at 37^{o}C in a 5% (v/v) CO_2 atmosphere in high-glucose Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, USA) supplemented with 10% fetal calf serum (Sigma-Aldrich, USA), 1% L-glutamine (Sigma-Aldrich, USA) and 1% Penicillin-Streptomycin (Life Technologies, USA).

2.3.2 MiRNA targeting MRPL28

Putative microRNAs targeting MRPL28 3'UTR were identified using the microRNA databases and target prediction tools miRWalk. MRPL28 (EntrezID 10573) was used as identifier. Computational predictive algorithms (miRanda, DIANAmT, miRWalk, RNAhybrid, and TargetScan) were employed to identify miRNA targets in 3'UTR of MRPL28 as well as the known miRNA target. Information on the location and the mature

miRNA sequence was identified with miRBase. DIANA miRPath v.2.0 was used to investigate the combinatorial effect of miRNAs in a pathway analysis (Vlachos et al, 2012).

2.3.3 Luciferase-reporter assays

HEK-293 cells ($4x10^4$) were transfected with pMIR-reporter plasmid (0.5µg, GeneCopeia, USA) containing a WT MRPL28 gene (pMIR-MRPL28) using Lipofectamine® 2000 (Invitrogen, USA) transfection reagent, followed by transfection with 16.7 pmol of different pre-miRNA (hsa-miR-18a-5p, hsa-miR-18b-5p, hsa-miR-19a-3p, hsa-miR-130a-3p, hsa-miR-130b-3p, hsa-miR-141-3p, hsa-miR-152-3p, hsa-miR-200a-3p, hsa-miR-324-3p, hsa-miR-326, hsa-miR-328-3p) precursor molecules. Luciferase activity was measured twenty-four hours after the last transfection with a luciferase assay kit (GeneCopeia, USA).

2.3.4 Transient transfections

2.3.4.1 siRNA against MRPL28

HEK-293 cells ($2x10^5$) were transfected with 15pmol and 30pmol Silencer®Select Pre-designed siRNA (Invitrogen, USA) against MRPL28 using Lipofectamine® 2000 (Invitrogen, USA) transfection reagent or Lipofectamine® RNAiMAX. Proteins were isolated by RIPA buffer with Protease Inhibitor Cocktail (sigma, USA) and analysis 24-72 hours after the last transfection. Proteins concentration was measured with Pierce^{TM}BCA Protein Assay Kit (Thermo Science, Norway).

2.3.4.2 miRNA transfection

HEK-293 cells ($4x10^4$) were transfected with 16.7pmol of pre-miRNAs (hsa-miR-18a-5p, hsa-miR-130b-3p, hsa-miR-326 and hsa-miR-328-3p) precursor molecules. After incubated in 37^{o}C incubator for 72 hours, proteins were isolated and analyzed by WB.

2.3.5 ETC chain composition and function

Relative protein expression of mitochondrial respiratory complexes I-V in HEK-293 was determined by WB using a Total OXPHOS Rodent WB Antibody Cocktail (Abcam, USA recognizing subunits from each of the five respiratory.

2.3.6 Western blotting analysis

Sample loading buffer and reducing buffer were added in to the lysate and samples from tissue. 20µg of solubilized proteins isolated by RIPA with protease inhibitor cocktail (10ml: 1 tablet) were loaded on 4-12% Bolt® Bis-Tris Plus gels (Life Technologies, USA). SeeBlue Plus2 Pre-stained Standard (Catalog no. LC5925) was loaded in the first lane as marker. After electrophorese the proteins were transferred into nitrocellulose membrane by iBlot Gel Transfer (Life Technologies, USA). Membranes were blocked with 0.1% Tween-20 and 5% BSA in PBS (or TBS) for 1 hour and then incubated overnight at 4^{o}C with primary antibodies, MRPL28 in 0.1% Tween-20 and 5% BSA in TBS or OXPHOS in 0.1% Tween-20 and 5% BSA in PBS. Rabbit monoclonal antibody against MRPL28 TA31098 (Origene Technologies), OXPHOS cocktail monopoly antibody (Mito-science, Norway), DAP3 (Abcam, USA), MRPL9 (Abcam, USA) were antibodies used to quantify the proteins. Membranes were also incubated with GAPDH antibodies (Sigma-Aldrich, USA), which is a normalization control. Membranes were washed with TBA-Tween with BSA and incubated in fluorescent-labeled secondary antibodies (LICOR Bioscience, UK) for 1.5 hours. Membranes were washed and then imaged by a high-resolution florescence scanner (Odyssey system, LI-COR Bioscience, UK). Images were analyzed by ImageStudio 2.0.

To optimization of the antibody and blocking buffer, three different type of blocking buffer: Odyssey blocking buffer, PBS with 5% of BSA, TBS with 5% BSA, PBS with 5% milk, TBS with 5% milk were tested as blocking buffer. Anti-MRPL28 antibody ab98922 (rabbit polyclonal to MRPL28, Abcam); Anti-MRPL28 QC24118 (Rabbit polyclonal, Sigma-Aldrich); Rabbit monoclonal antibody against MRPL28 (TA31098, Origene Technologies) were tested in different blocking buffer.

2.4 *In vivo* experiments

2.4.1 Tissue sample

Animals were scarified by cervical dislocation. Aorta, brain, heart (free wall of the left ventricle), kidney, liver, lung, ovary, soleus and testis were collected from health adult C57BL/6 mice. In addition, skeletal muscle (soleus) and heart tissues (free wall of the left ventricle) from HCR and LCR rats were collected. The HCR and LCR animals are from generation 30, at 9-10 months of age. The tissues were put into liquid nitrogen and immediately after collection and kept in -80^{o}C. 20-40 mg of the tissues were pulverized and homogenized in lysis buffer (RIPA buffer with 1 tablet protease inhibitor cocktail) several times with 5 minutes interval each time until there was no visible fragment. After centrifugation at 13000xg 4^{o}C for 20 minutes, supernatant with solubilized proteins was collected. Proteins concentration was measured with Pierce^{TM}BCA Protein Assay Kit. Samples were added with reducing buffer and LDS buffer and analyzed by WB.

2.4.2 MRPL28 expression level in pathological status

MRPL28 expression level was evaluated in plantaris muscle of Wistar rats before and after HIT. Wistar rat were injected with ~10^{6} Walker 256 tumor cells and in the bone marrow by an osteotomy in femur (n=5). Wistar rats displayed >10% of mass reduction. Control rats were submitted to sham osteotomy (n=5). Rats were killed 12 days post tumor cells injection (or sham osteotomy) and the plantaris muscle was stored at -80^{o}C to proteomic analysis. Briefly, gel-free and label-free proteomics were performed (Abdallah et al, 2012).

2.4.3 Cardiac miRNAs in LCR and HCR rats

Microarray assay for miRNAs was performed the LV sample of HCR (n=5) and LCR (n=5) rats using a service provider (Exiqon, Denmark). The assay started with 4–8 μg total RNA sample. The detection probes were made by in situ synthesis using photo-generated reagent chemistry. Hybridization used 100 μL 6× SSPE buffer (0.90 M NaCl, 60 mM Na2HPO4, 6 mM EDTA, pH 6.8) containing 25% formamide at 34°C. After RNA hybridization, tag-

conjugating Cy3 or Cy5 dyes will be circulated through the microfluidic chip for dye staining. Fluorescence images were collected using a laser scanner and digitized using Array-Pro image analysis software.

2.5 Statistical analysis

We used a one-way analysis of variance (ANOVA) to compare three or more groups/conditions, followed by Tukey multiple comparison test. When two groups were compared, a one tail unpaired t-test was used. P value below 0.05 was considered significantly.

2.6 URLs

ECRs: http://ecrbrowser.dcode.org;

miRBase: http://microrna.sanger.ac.uk/;

miRWalk: http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/;

SNPexp (version 1.2): http://app3.titan.uio.no/biotools/tool.php?app=snpexp;

Diana Tools: http://diana.imis.athena-innovation.gr/DianaTools/index.php;

EMBL-EBI Protein Data Bank in Europe: http://www.ebi.ac.uk/pdbe/

Gene Expresion Omnibus: http://www.ncbi.nlm.nih.gov/geo/

3. RESULTS

3.1 Population study

3.1.1 Bioinformatics approaches to select SNPs association for VO_{2max}

MRPL28 variants were interrogated for association with VO_{2max} in the HUNT fitness study and a significantly association were identified between rs3830160 and VO_{2max} (P=0.02215, adjusted for age, physical activity and body mass index) (Figure 2). Interestingly, an allelic effect on the oxygen uptake values was observed for the rs3830160 variant. Individuals carrying the GG genotype presented significantly lower VO_{2max} levels compared to the AA genotype (1.39±0.02 vs. 1.37±0.01 vs. 1.34±0.01* $mLO_2 \cdot kg^{-1} \cdot min^{-1}$, *P=0.0269 compared to AA, for AA, AG and GG genotypes, respectively) (Figure 2B). Interestingly, bioinformatics analysis using SNPexp tool revealed that rs383016 variant also influence MRPL28 gene expression significantly (Figure 2C and 2D). MRPL28 rs3830160 genotypes are significantly association with MRPL28 gene expression level in all population together ($P=2.638x10^{-13}$) and in CEU (P=0.001355), CHB (P=0.006779), JPT (P=0.000391) but not significantly association in YRI (0.7406) (Table 2). When all populations are analyzed together, the individuals carrying the rs3830160 GG genotype presented significantly lower MRPL28 mRNA levels compared to the AA genotype (10.66±0.27 vs. 10.47±0.29 vs. 10.27±0.27*, *P<0.005 compared to AA, for AA, AG and GG genotypes, respectively)(Figure 2C).

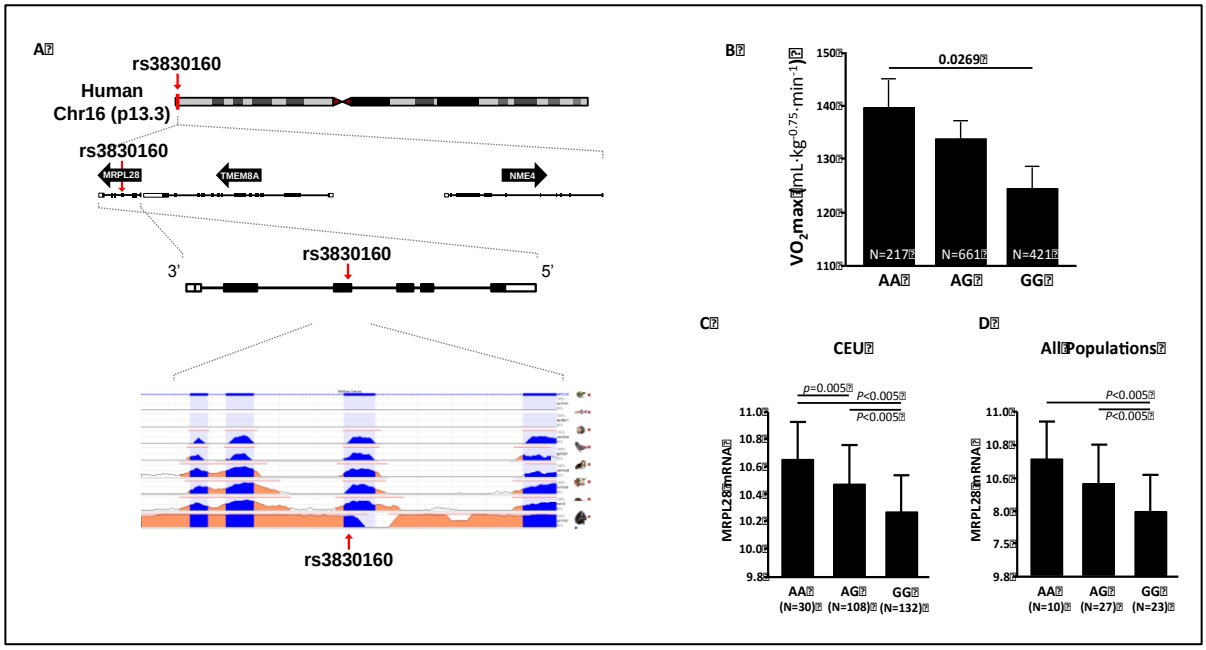

Figure 2. MRPL28 rs3830160 variant influences VO_{2max} levels and MRPL28 gene expression. A. A evolutionary conserved region, on human chromosome 16p13.3 location of miRNA target site SNP (rs3830160), associated with VO_{2max}. From the bottom to the top the reference genome (*Homo sapiens*) is aligned against chimpanzee (*Pan troglodytes*), cow (*Bos Taurus*), mouse (*Mus musculus*), dog (*Canis familiaris*), chicken (*Gallus gallus*), Opossum (*Monodelphis domestica*), frog (*Xenopus laevis*) and zebrafish (*Danio rerio*) genomes (Ovcharenko et al, 2004). The conserved region varies from 97.4% comparing the human sequence with the chimpanzee to 71.5% with the Opossum. B. Maximum oxygen uptake (VO_{2max}) distribution, according to the genotype (AA, AG, or GG), for rs3830160 variant in the HUNT Fitness population. Note that the presence of allele G lead to a decrease in the aerobic capacity in this population. MRPL28 expression levels according to the rs3830160 genotype in an European population (panel C) and in all populations (panel D).

Table 2. Role of MRPL28 rs3830160 variant in gene expression (SNPexp v 1.2).

Population	CHR	SNP	BP	A1	TEST	NMISS	BETA	STAT	P value
All	16	rs3830160	359093	A	ADD	270	0.1921	7.7	$2.638x10^{-13}$
CEU	16	rs3830160	359093	A	ADD	60	0.1838	3.367	0.001355
CHB	16	rs3830160	359093	A	ADD	45	0.1971	2.845	0.006779
JPT	16	rs3830160	359093	G	ADD	45	-0.2611	-3.847	0.000391
YRI	16	rs3830160	359093	A	ADD	60	-0.02407	-0.333	0.7406

CEU: Utah residents with ancestry from northern and western Europe; CHB: Han Chinese in Beijing; JPT: Japanese in Tokyo; YRI: Yoruba in Ibadan

3.1.2 rs3830160 allele distribution in different populations

In order to test for allele variations in different ethical populations, we have used a web tool (SNPexp) for calculating and visualizing correlation between HapMap genotypes (Genome-wide SNP genotyping in 270 individuals from 6 populations) (Holm et al, 2010) and gene expression levels for MRPL28. rs3830160 variant presents similar distribution in the allele frequency in the world except in the Japanese (JPT) population. In the world all population (A allele, 39.5%; G allele, 60.5%), CHB population (A allele 35.5%, G allele64.5%), and YRI (A allele, 37.2%, G allele, 62.8%) A allele is more frequent allele. In JPT (A, 54.5% G, 45.5%) population A and G allele present a comparably balance distribution but high frequency of G allele, which is conversely distributed, compared to other population. There is no obviously geography differentiation distribution (Table 3).

Table 3. MRPL28 rs3830160 allele population distribution in all population (SNPexpv1.2).

Population	rs3830160 allele	
	A	G
All populations	168 (31%)	372 (69%)
CEU	47 (39.5%)	73 (60.5%)
CHB	32 (35.5%)	58 (64.5%)
JPT	49 (54.5%)	41 (45.5%)
YRI	29 (37.2%)	49 (62.8%)

3.1.3 MRPL28 expression in pathological conditions

HUNT-MI Study has genotyped 80,137 coding variants in 5,643 Norwegians. Using this large cohort of the HUNT database, the SNPs located in MRPL28 exons were evaluated associated with myocardial infarction (MI) (Holmen et al, 2014). SNPs, rs114486517 have a tendency to associate with the genotype MI. But the association did not reach the significant level. No information of rs3830160 was detected in HUNT MI study (Table 4).

Table 4 MRPL28 SNPs associated with myocardial infarcted (MI) patients from the HUNT-MI study.

rsID	Exomchip ID	BP	A1	F_A	F_U	A2	CHISQ	P value	OR
rs114486517	exm1196139	417691	G	0.001553	0.001549	C	4.33e-05	0.9947	1.003
rs141083967	exm1196142	420208	ND						
rs147315737	exm1196149	417722	ND						
rs149440376	exm1858177	420043	ND						
rs150828940	exm1858180	418348	ND						
rs181590179	exm1196213	418381	ND						
rs200752498	exm1196215	420056	ND						
rs201250294	exm1196218	418527	ND						
rs3194151	exm1196224	420140	ND						
rs80158709	exm1196232	420079	A	0.1446	0.1325	G	3.587	0.05822	1.107

ND, non-detected in the HUNT MI Study.

3.2 *In vitro* experiments

In order to further characterize MRPL28 role, we here describe the different in vitro approaches performed using cell lines. miRNAs putative targeting MRPL28 will be determined by bioinformatics method and validated by luciferase assay. MRPL28 will be transient transfected by either siRNA or miRNA in HEK-293 cells and OXPHOS compositions will be tested before and after transfection.

3.2.1 MiRNAs predicted to target MRPL28

Putative miRNAs targeting MRPL28 3'UTR were identified using the target prediction tool miRWalk. A total number of 11 miRNAs (hsa-miR-18a-5p, hsa-miR-18b-5p, hsa-miR-19a-3p, hsa-miR-130a-3p, hsa-miR-130b-3p, hsa-miR-141-3p, hsa-miR-152-3p, hsa-miR-200a-3p, hsa-miR-324-3p, hsa-miR-326 and hsa-miR-328-3p) were identified by a combination of five different predictive algorithms (DIANAmT, miRanda, miRWalk, RNAhybrid and TargetScan) (Table 5). It was considered only the miRNAs predicted to targeting MRPL28 3'UTR identified by all five algorithms.

Table 5. Predicted miRNA binding sites on MRPL28 mRNA 3'UTR in common to 5 different algorithm (DIANAmT, miRanda, miRWalk, RNAhybrid and Targetscan).

Accession	microRNA	Chr location	Seed region (MRPL28 3'UTR)	
			Start	End
MI0000804	hsa-miR-328	chr16: 67202321-67202395	1097	1091
MI0000808	hsa-miR-326	chr11: 75335092-75335186	1017	1009
MI0000072	hsa-miR-18a	chr13: 91350751-91350821	963	955
MI0000448	hsa-miR-130a	chr11: 57641198-57641286	882	875
MI0000457	hsa-miR-141	chr12: 6964097-6964191	1055	1049
MI0000813	hsa-miR-324	chr17: 7223297-7223379	926	920
MI0000073	hsa-miR-19a	chr13: 91350891-91350972	880	874
MI0000462	hsa-miR-152	chr17: 48037161-48037247	883	876
MI0001518	hsa-miR-18b	chrX: 134170041-134170111	963	955
MI0000737	hsa-miR-200a	chr1: 1167863-1167952	1055	1049
MI0000748	hsa-miR-130b	chr22: 21653304-21653385	882	875

3.2.2 Luciferase reporter gene assay

On the basis of the findings in the last step, the functionality of the seed regions for MRPL28 3'UTR was tested using a luciferase reporter gene assay. miRNA expression reporter constructs were generated by adding wild type 3'UTR region of MRPL28 under post-translational region of firefly luciferase gene. Co-transfection of HEK-293 cells with synthetic precursors for every single miRNA decreased MRPL28 3'UTR reporter activity for hsa-miR-18a-5p, hsa-miR-130b-3p, hsa-miR-326 and hsa-miR-328-3p, whereas no change of control miRNA transfection was observed (Figure 3). KEGG pathway analysis showed that hsa-miR-18a-5p, hsa-miR-130b-3p, hsa-miR-326 and hsa-miR-328-3p are commonly enriched in pathway in early embryonic development, cancer's pathway and pyruvate metabolism pathway which is an important pathway to mitochondrial energy generation (Table 6) (Gray et al, 2014).

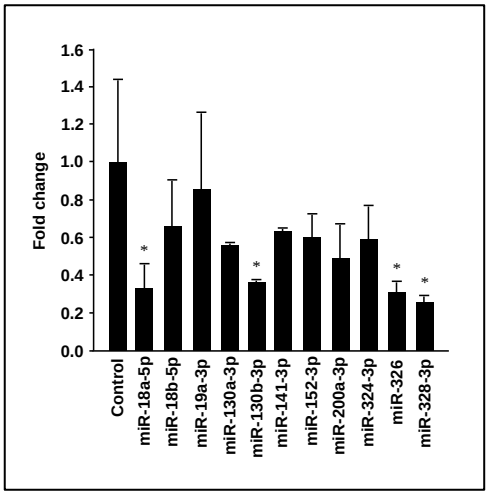

Figure 3. Luciferase assay for miRNA validation. HEK-293 cells were transfected with synthetic precursors of mi-RNA against MRPL28 and constructor. Co-transfection of HEK-293 cells with synthetic precursors for every single miRNA decreased MRPL28 3'UTR reporter activity for hsa-miR-18a-5p, hsa-miR-130b-3p, hsa-miR-326 and hsa-miR-328-3p significantly, whereas no change of control miRNA transfection was observed. *P<0.05 compared to Control.

Table 6. KEGG pathway analysis for hsa-miR-328, hsa-miR-326-3p, hsa-miR-18a-5p and hsa-miR-130b-3p.

KEGG pathway	**P-value**
Prion diseases (hsa05020)	$1.79x10^{-44}$
Pathways in cancer (hsa05200)	$4.18x10^{-06}$
Hedgehog signaling pathway (hsa04340)	$2.38x10^{-05}$
Endocytosis (hsa04144)	$2.28x10^{-04}$
Colorectal cancer (hsa05210)	$2.22x10^{-03}$
Prostate cancer (hsa05215)	$2.22x10^{-03}$
mTOR signaling pathway (hsa04150)	$4.43x10^{-03}$
RNA degradation (hsa03018)	$9.66x10^{-03}$
Basal cell carcinoma (hsa05217)	$9.66x10^{-03}$
Bladder cancer (hsa052199)	$1.00x10^{-02}$
Cell cycle (hsa04110)	$1.09x10^{-02}$
Chronic myeloid leukemia (hsa05220)	$1.09x10^{-02}$
TGF-beta signaling pathway (hsa04350)	$1.14x10^{-02}$
Chronic myeloid leukemia (hsa05220)	$1.09x10^{-02}$
TGF-beta signaling pathway (hsa04350)	$1.14x10^{-02}$
Pancreatic cancer (hsa05212)	$1.43x10^{-02}$
Vasopressin-regulated water reabsorption	$1.82x10^{-02}$
Terpenoid backbone biosynthesis (hsa00900)	$2.42x10^{-02}$
mRNA surveillance pathway (hsa03015)	$2.54x10^{-02}$
Lysine degradation (hsa00310)	$2.72x10^{-02}$
Glioma (hsa05214)	$3.12x10^{-02}$
Transcriptional misregulation in cancer (hsa05202)	$3.38x10^{-02}$
Non-small cell lung cancer (hsa05223)	$3.93x10^{-02}$
Pyruvate metabolism (hsa00620)	$3.93x10^{-02}$

3.2.3 Transient transfection of siRNA against MRPL28 or with miRNA targeting MRPL28 in cell line

HEK-293 cells ($2x10^5$) were transfected with Silencer®Select Pre-designed siRNA (Invitrogen, USA) against MRPL28 using Lipofectamine® 2000 (Invitrogen, USA) transfection reagent or Lipofectamine® RNAiMAX several times with different concentration and transfection time. Proteins isolated from normal HEK-293 cells (normal control group) and MRPL28 were analysis by WB. There are no significantly different in the expression of MRPL28 compared with control group in which the HEK-293 cells were only transfected with Lipofectamine (Figure 4).

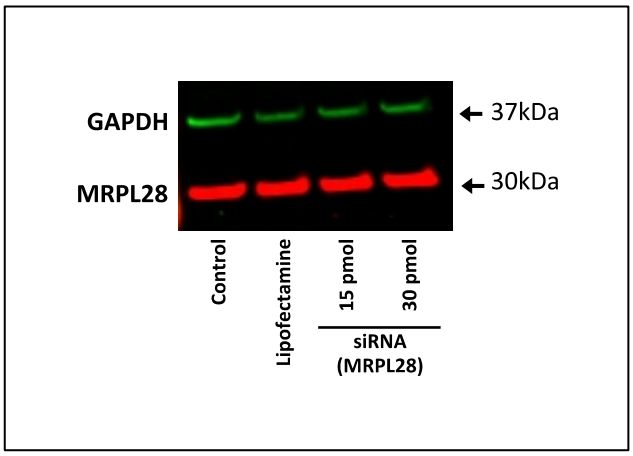

Figure 4. Transient loss-of-function (siRNA) of MRPL28 in HEK-293 cells. 15 pmol and 30 pmol of siRNA against MRPL28 were transfected into HEK-293 cells. Compared to the control group and the group only transfected with Lipofectamine® RNAiMAX, MRPL28 level has no significantly change.

HEK-293 cells ($4x10^4$) were transfected with 16.7pmol of pre-miRNAs (hsa-miR-18a-5p, hsa-miR-130b-3p, hsa-miR-326 and hsa-miR-328-3p) precursor molecules. After incubated in 37^0C incubator for 72 hours, proteins were isolated and analyzed by WB. MRPL28 expression level was reduced in miRNA transfection group compared with normal control group. The highest reduction was induced by hsa-miR-328 transfection (30% reduced) (Figure 5).

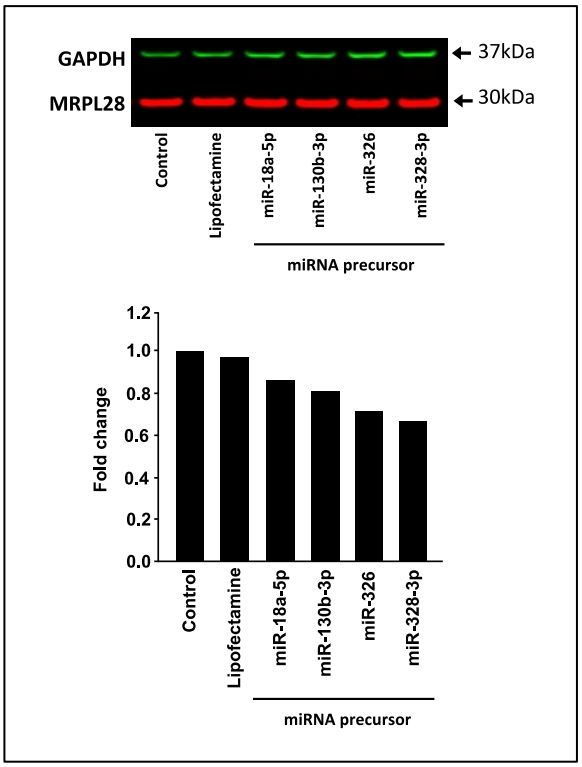

Figure 5. miRNA precursor transient transfection in HEK-293 cells. HEK-293 cells were transfected by pre-miRNAs (hsa-miR-18a-5p, hsa-miR-130b-3p, hsa-miR-326 and hsa-miR-328-3p). MRPL28 expression level was reduced in miRNA transfection group compared with normal control group.

3.2.4 OXPHOS compositions evaluation

Relative proteins expression of mitochondrial respiratory complexes I–V in HEK-293 were determined by WB using a cocktail of antibodies that recognizing subunits from each of the five respiratory complexes. The cocktail of antibodies contains 5 monoclonal antibodies, one each against Complex I subunit NDUFB8 (MS105), Complex II-30kDa (MS203), Complex III-Core protein 2 (MS304) Complex IV subunit I (MS404) and CV alpha subunit (ATP5A). From the WB result, complex V, complex III and complex II were identified with clearly bands. There is no regularly a change was found after transfection HEK293 with miRNA (Figure 6).

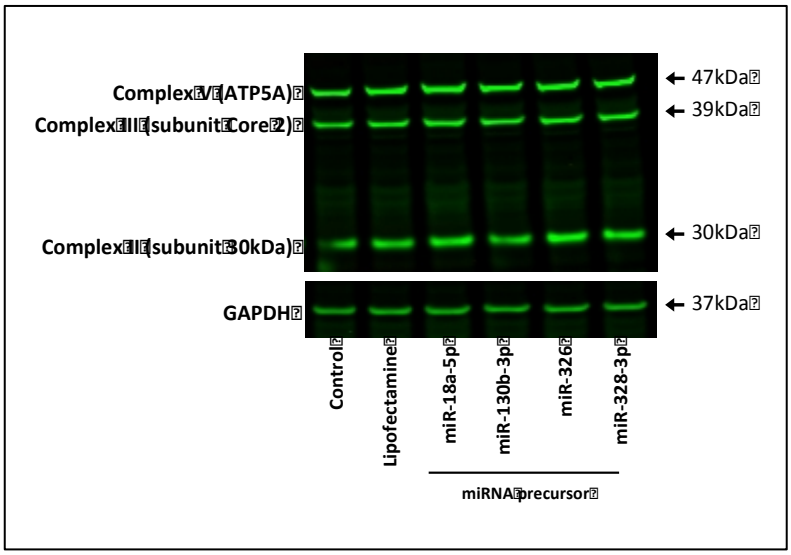

Figure 6. Changes in the OXPHOS induced by transient transfection of HEK-293 cells with miRNA precursors. HEK-293 cells were transfected by pre-miRNAs (hsa-miR-18a-5p, hsa-miR-130b-3p, hsa-miR-326 and hsa-miR-328-3p). OXPHOS compositions were evaluated after transfection. No significantly difference was identified in complex V, III and II. Complex I and IV were not detected.

3.3 *In Vivo* experiments

3.3.1 MRPL28 tissue distribution via western blot analysis

MRPL28 tissue distribution was analyzed in samples collected form adult C57BL/6 mice. A total of 20 μg of protein lysate from aorta, brain, heart (left ventricle), kidney, liver, lung, ovary, soles and testis from health mice were analysis by WB for MRPL28 (Figure 7). MRPL28 distributed diversely in all the tissues collected in this study. MRPL28 especially highly expressed in kidney, brain and lung and testis. MRPL28 expression level is similarly in soleus and heart and is approximately 40% of MRPL28 in kidney.

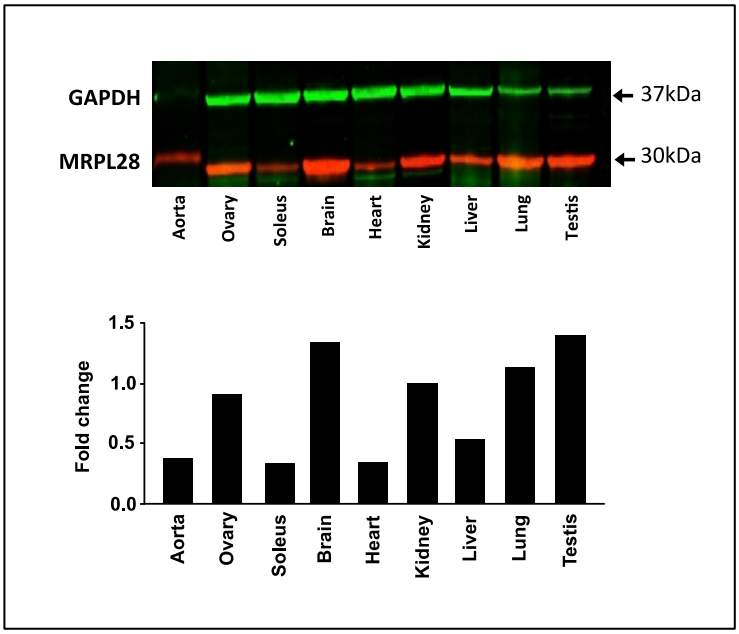

Figure 7. MRPL28 tissue distribution in adult mice. MRPL28 level was tested in different tissues from adult C57BL/6 mice. MRPL28 has comparatively high level expression in kidney, brain, lung testis.

3.3.2 MRPL28 expression in experimental pathological conditions

MRPL28 expression level was evaluated in plantaris muscle of Wistar rats before and after HIT. There is no significantly change of MRPL28 level in tumor group than control group before and after HIT. MRPL28 was not detected in B16 animal models (Table 7)

Table7. MRPL28 expression in cancer models (Walker and B16).

Tumor Types	Groups		
	Control	Tumor	Tumor + HIT
Walker (rats)	14.0±0.7	13.6±0.7	11.6±2.6
B16	ND	ND	ND

HIT, high-intensity interval training; ND, on-detected.

3.3.3 MRPL28 expression in physiological conditions

Skeletal muscle (n=8) and heart tissue (left ventricular) (n=14) from LCR/HCR (generation 30, at 9-10 months of age) were collected and analysis by WB. MRPL28 protein level in soleus is no significantly difference in HCR and LCR. MRPL28 protein level in LV was significantly higher (1.78 fold) in HCR than LCR. Correlation between MRPL28 level and both running capacity and VO_{2max} were performed. There is no significant correlation between MRPL28 and running capacity or VO_{2max} (Figure 8).

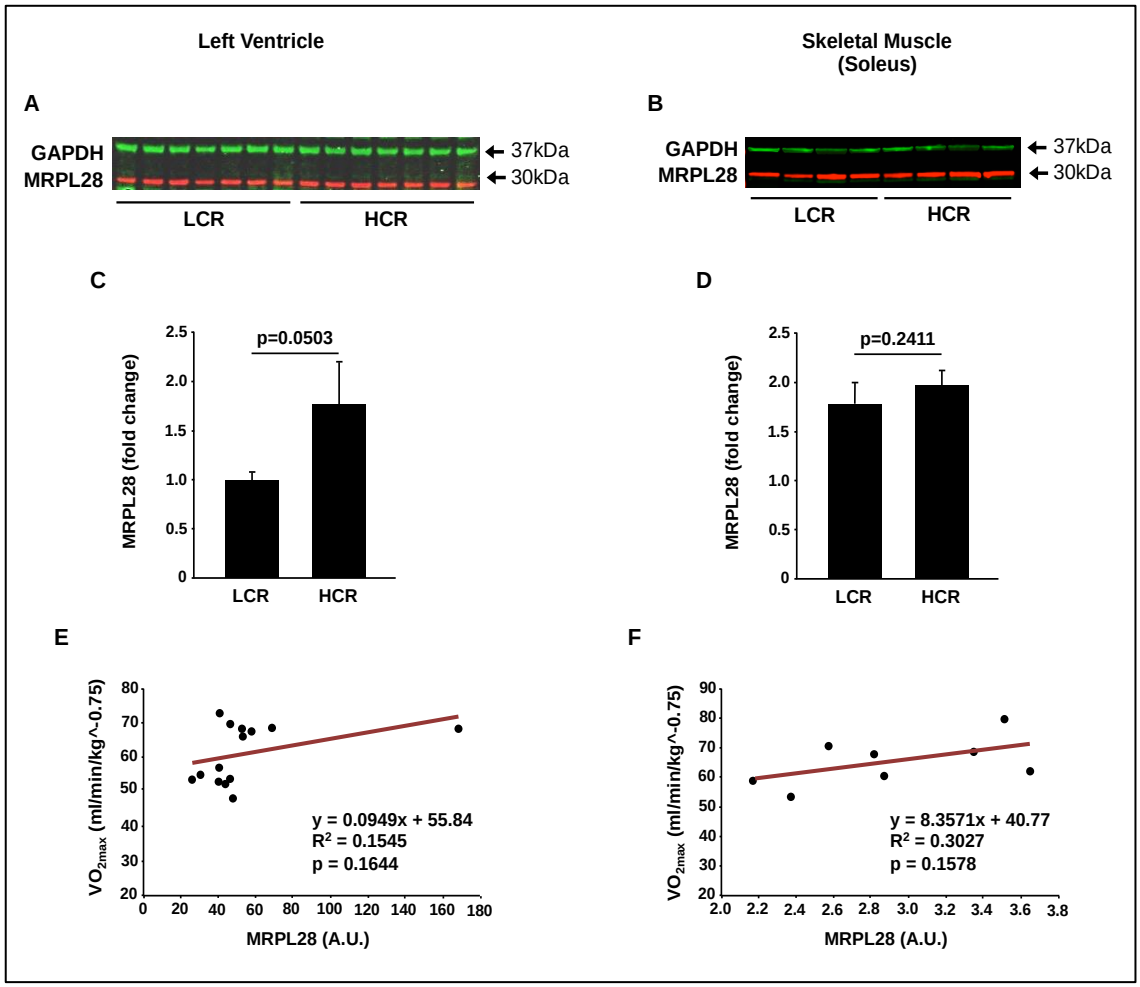

Figure 8. MRPL28 expression level in HCR/ LCR mice. Panel A, MRPL28 level in LV from HCR (lane 8-14) was 1.7 fold higher than from LCR (lane1-7); C and E showed there was no significantly correlation between MRPL28 and running capacity or VO_{2max}. B, MRPL28 level in soleus from LCR (lane1-7) and HCR (lane 8-14) was no significantly different; D and F showed there was no significantly correlation between MRPL28 and running capacity or VO_{2max}.

3.3.4 Cardiac miRNAs in LCR/HCR rats

miRNA array of the sample from HCR and LCR animals was gained in previous study in CERG. miRNAs target MRPL28 were checked in this data. miR-18a-5p was not detected in this study. Hsa-miR-130b-3p, hsa-miR-326, hsa-miR-328-3p levels were not significantly different in LV tissue from LCR and HCR rats (Table 8).

Table 8. Cardiac miRNAs target MRPL28 in HCR/LCR rats.

miRNA	**LCR**	**HCR**	**P-Value**
rno-miR-18a-5p	ND	ND	ND
rno-miR-130b-3p	0.225	0.314	0.384
rno-miR-326	-0.312	-0.233	0.489
rno-miR-328-3p	0,195	0,269	0.633

ND, non-detected in the array.

4. DISCUSSIONS

Several findings were demonstrated in this study: first, a common variant for MRPL28 (rs3830160) was identified significantly association with VO_{2max}; second, other variants in MRPL28 was not associated with myocardial infarction in patients from the HUNT-MI Study; third, MRPL28 is ubiquitous expressed in virtually all tissues and organs in mice; fourth, MRPL28 expression level in LV was 1.78 fold higher in HCR than LCR rats; fifth, MRPL28 protein levels in the skeletal muscle were not different detected in two independent cancer cachexia rodents models; sixth, MRPL28 is target by several miRNAs (i.e., hsa-miR-18a-5p, hsa-miR-130b-3p, hsa-miR-326 and hsa-miR-328-3p).

We have demonstrated for the first time that MRPL28 is a candidate gene for aerobic capacity. Previously, we have reported that rs540 variant, located in a non-coding region of TMEM8A, was also associated with aerobic capacity (unpublished data). The meticulous analysis of the human sequence for this locus showed that rs3830160 is located in a highly conserved region when compared with the sequence in different species, sharing sequence with Opossum, chicken, dog, mouse, cow, and chimpanzee. By comparing the genomic sequence in the vicinity of the MRPL28 rs3830160 variant from species representing different evolutionary clades, such as primates and fish, we assessed evolutionary fates shared by all vertebrates. Interestingly, human MRPL28 is positioned in a long syntenic region (Chr16:367384-370569), shared with mouse (Chr17:26123503-26126613), rat (Chr10:15309316-15312227) in the cluster of the α-globin genes, which is responsible for oxygen transport and delivery. This chromosomal region seems to contain critical cis-acting elements required for the appropriate regulation of the α-like globin genes expression. Although it is not clear if MRPL28 can influence oxygen uptake by playing a role in the globin pathway, there are some evidences suggesting that TMEM8A is an important player. In cultured erythroblasts, Philonenko et al. (Philonenko et al, 2009) were able to show that TMEM8A directly interacts with the downstream enhancer element (CpG site) of the α-globin gene domain suggesting that partner regulatory elements might be located within these regions. Interestingly, TMEM8A is not recruited to the α-globin gene domain active chromatin hub. Instead, an alternative chromatin hub is assembled, including some of the regulatory elements essential for the activation of globin gene expression.

Several are the SNPs identified to be associated with aerobic capacity such as variants in the ACE (Angiotensin I converting enzyme), LEPR (leptin receptor) and MCR4 (melanocortin receptor), PAPSS2 (3'-phosphoadenosine 5'-phosphosulfate synthase 2) genes (Bouchard et al, 2011a). rs6552828 located in the acyl-CoA synthase long-chain member 1 (ACSL1) gene and other 21 SNPs were identified associated with gain of VO_{2max} after standardized 20-wk exercise (Bouchard et al, 2011b). Most of the genes are association with glucose and fat metabolism and energy production. Our group has also identified other 5 variants (rs3803357, rs6950857, rs10494973, rs2679066 and rs1938593) associated with aerobic capacity in the HUNT Fitness population and validated in another Cohort (Generation 100 Study) (unpublished data). Here we also tested if SNPs located in the MRPL28 gene were association in myocardial infarcted (MI) patients from the HUNT-MI study, but we could not find any association. However, several recent clinical cases reported that mutations in MRPs (MRPL3, MRPL44, MRPS16, and MRPS22) caused series clinical symptoms with the common heart symptom: hypertrophic cardiomyopathy (HCM) (Carroll et al, 2013; Galmiche et al, 2011; Miller et al, 2004; Smits et al, 2011). HCM is an original or compensation for the overload of heart which is a risk factor to both sudden death and progressive systolic heart failure (Yacoub et al, 2007). All the cases reported with mutation in MRPs had congenital HCM, which might suggest that MRPs play important role in heart development or play role in cardiac remodeling. All the patients with mutation in MRPs also had tissue specific deficiency in oxidative phosphorylation and reduction of the mitochondrial production in respiratory chain in complex I, III or IV in heart, skeletal muscles or fibroblast (Carroll et al, 2013; Galmiche et al, 2011; Saada et al, 2007; Smits et al, 2011). We did not detect any changes in complex II, III and V after transient transfection of MRPL28 in HEK-293 cells in vitro. But knock down of MRPL28 was identified reduce expression of complex III and decrease mitochondrial oxygen consumption of cells (SU86 and Miapaca2 pancreatic cancer cells) in vitro and more reduction of MRPL28 induced more reduction of mitochondrial oxygen consumption (Chen et al, 2009). MRPL28 might modify mitochondrial function and further influence mitochondria associated phenotypes. However, many compensation and interaction in physiological and pathological process would make it more complicated to discovery the association between the genotype and phenotype.

In order to further describe physiological and pathological roles for MRPL28, we made use of different experimental models available in our laboratory. MRPL28 was not detected significantly different in the skeletal muscle of a Cancer cachexia rat model (Walker 256 tumor). It has been demonstrated that loss of MRPL28 in pancreatic tumor cells could increase the growth rate of tumor in vivo but knock down of MRPL28 would decrease the tumor cells growth rate in vitro (Chen et al, 2009). Regarding the physiological models for acquire aerobic capacity, we found that MRPL28 was 1.78 fold higher in HCR than LCR animal models. Recently, a proteomic analysis also revealed that nearly all of MRPs including MRPL28 were significantly different from LCR and HCR skeletal muscle, extensor *digitorum longus* muscles (EDL) tissue (Overmyer et al, 2015). The paper suggested that high efficient of mitochondria utilization of BBCA and fat during exercise contributed on high oxygen capacity (Overmyer et al, 2015). Although we have not tested is MRPL28 could influence other phenotypes, such as longevity, there are strong evidences in the literature showing the several members of the MRPs family play an important role prolonging lifespan. A longevity study identified that Mrps5 and other MRPs were associated with lifespan of BXD (a recombination strains of mice) and knockdown of Mrps5 and MRPs decreased mean lifespans of *C. elegans* (Houtkooper et al, 2013). This study also suggested knockdown of Mrps5 and other mitochondrial ribosomal proteins might triggers mitonuclear protein imbalance, reducing mitochondrial respiration and activating the mitochondrial unfolded protein response (Houtkooper et al, 2013). Interestingly, the increase in the MRPL28 levels (as well as MRPS5) observed in the HCR animals, is accompanied by higher aerobic capacity (VO_{2max}) levels, lower CVDs risk factors and superior lifespan (Koch et al, 2011).

Several post-transcriptional mechanisms, such as miRNAs, can control gene expression. MiRNAs expression in the cardiovascular system were identified associated with many pathological process of CVDs and physiological statue such as exercise (Flowers et al, 2015; Ono et al, 2011). Implication of miRNA which is an important post-translation regulator in CVD as potential target therapeutic function and early diagnosis biomarkers was recognized in recent year (Small et al, 2010). miR-210, miR-21 and miR-222 were identified increased in healthy subjects with low VO_{2max} in this study (Bye et al, 2013). Each miRNA regulate many mRNAs and each mRNA is also regulated by many miRNAs at the same time (Ono et al, 2011). Here, we were able to demonstrate that several putative miRNAs were predicted by bioinformatics to target MRPL28. In this study we showed that miR18a, miR130b, miR326

and miR-328 could target MRPL28 and down regulation of MRPL28 in vitro. In anther study, miR-130b was also identified suppressed fat deposition, which is important factor for CVDs by inhibiting expression of PPAR-gene in cell line (Pan et al, 2013). The same research group revealed that microvesicle-shuttled miR-130b reduces fat deposition in primary porcine adipocytes (Pan et al, 2014). A genome wide study on miRNA expression association with rat pulmonary artery revealed that miR-328 was drastically down regulated in the pulmonary artery (PA) after a hypoxic assault and overexpression miR-328 remarkably decreased the right ventricular systolic pressure and PA wall thickness under both normoxia and hypoxia in rat (Guo et al, 2012). Above all, miRNAs regulate MRPL28 also take part in many process associated CVDs.

5. CONCLUSION

Taken together, MRPL28 is a promising candidate target for aerobic capacity which is an important predictor of mortality for both CVD and health population. The identification and validation of new aerobic capacity genetic variants will bring forward a potential to establish original and novel diagnostic and therapeutic strategies for cardiovascular disease (CVD) management, thus contributing to reduce the burden of cardiovascular and life-style related diseases. Appropriate exercise training can develop aerobic capacity significantly. Exercise training could decrease both risk factor for CVD and for many other diseases through develop aerobic capacity, and improve lift quality for both CVD patients and health population through many mechanisms.

6. FUTURE PERSPECTIVES

6.1 Drosophila as a model organism to study aerobic capacity

Because the conservation of the basic gen regulator system, some organism like flies, worms, and fish can be used to investigate the underlying mechanisms driving heart development and function. Drosophila (fruit fly *D. melanogaster*) as a simplest genetic model with fluid pumping heart was validated as an *in vivo* model for analyzing candidate genes involved in cardiac function (Bier & Bodmer, 2004). At the same time, drosophila was utilized to be animal model for mitochondrial diseases (Garesse & Kaguni, 2005; Guo, 2012) and aging because drosophila has sequenced nuclear and mitochondrial genomes. Moreover, many mutations in conserved genetic pathways have been found and multiple genetic tools are available for drosophila (Morrow & Tanguay, 2008). It is reasonable to use drosophila to study MRPs, which were identified involving in CVD and aging process mediated by mitochondrial function. In fact, our group recently started to collaborate with Prof Rolf Bodmer from the Sanford-Burnham Medical Research Institute (La Jolla-USA) to use the fruit fly *D. melanogaster* as a model organism to investigate candidate genes and their role in the heart structure and function. More recently, we got a grant proposal funded by UNIKARD entitled «Developing a Collaborative Platform for the Use of the *Drosophila Melanogaster* as Model Organism to Understand Heart Ageing and Longevity». These are on-going project that will help us to unveil the role of MRPL28 in the cardiovascular system.

6.2 Genetically modified animal models

With the development of the genome manipulation, permanent and transient modification of an interested gene becomes feasible. Utilization of the gene modification animal models can lead to better understanding of physiological and pathological network ensued by a specific modified gene. Rodents are mammalian with 4-chambered heart has been the choice for generate transgenic model of CVD (Yutzey & Robbins, 2007). MRPL28 and other MRPs in the complex physiology network and environmental factors could be studied in transgenic animals in the future. In December 2013, we have been selected by The Medical College of Wisconsin (MCW) Gene Editing Rat Resource Center, coordinated by Prof. Aron Geurts, for

the production of a mutant rat for MRPL28 (with a Lewis rat strain background) with resources from the National Heart, Lung, and Blood Institute (NHLBI, R24 Resource grant). The mutant rat for MRPL28 was successfully developed during 2014 and should be shipped to our group just after this summer. Simultaneously, we started collaboration with the group of Prof Håvard Attramadal, Faculty of Medicine-University of Oslo-Norway, for the production of a transgenic mouse with cardiac restricted overexpression of Mrpl28 from C57BL/6 mice. The production of the MRPL28 cardiac specific TG mice is still on going and the expectation is to have both animal models ready to be shipped to our Animal Core Facility in 2016. Therefore, we will use both gain- and loss-of-function strategies to address the MRPL28 role *in vivo* by using dedicated animal models.

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