UNIVERSITI PUTRA MALAYSIA

TRANSCRIPTOMIC AND PROTEIN EXPRESSION ANALYSES OF SKELETAL MUSCLES ISOLATED FROM Ts1Cje MOUSE MODEL FOR DOWN SYNDROME

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FPSK(M) 2017 51

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

May 2017

Abstract of thesis presented to the senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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May 2017

Chairman : Ling King Hwa, PhD
Faculty : Medicine and Health Sciences

Down Syndrome (DS) is caused by an additional copy of human chromosome 21 (HSA21). It is characterised by several clinical phenotypes such as intellectual disability, facial features, early onset dementia, weak immune system and hypotonia. Deficits in motor functions happens throughout development among DS individuals leading to muscle weakness, joint hyperextensibility, delayed acquisition of postural control and poor balance. To date, the underlying cause of hypotonia in DS individuals remains unknown and limited studies on the pathophysiological and molecular characterisation of hypotonia in DS could be found. Ts1Cje is a DS mouse model with partial trisomy of mouse chromosome 16 (MMU16), which encompasses a high number of HSA21 orthologous genes. This mouse model has been well reported to exhibit various typical neuropathological features as well characterised with muscle weakness that is similar to individuals with DS. The hypothesis of this study is that the trisomic genes in MMU16 are over-expressed in Ts1Cje mice and disrupts the functional molecular networks, leading to muscle weakness in Ts1Cje. To test this hypothesis, the study was divided into two major parts namely the targeted approach to study selected important markers in muscle development and function, and global transcriptomic gene expression study to identify novel genes involved in Ts1Cje muscle weakness. In this study, the soleus and extensor digitorum longus (EDL) skeletal muscles were harvested from both Ts1Cje mice and disomic control mice. Reverse transcription quantitative real time polymerase chain reaction (RT-qPCR) analysis of nine selected genes *Lamc1, Leprel1, Myl6b, Msn, Pgm5, Tmod1, Istn1, Synj1* and *Rcan1* showed an upregulation of the triplicated genes *Itsn1, Synj1* and *Rcan1* in both soleus and EDL muscles of the Ts1Cje mice. The disomic genes *Lamc1, Leprel1, Msn, Pgm5* and *Tmod1* did not show any significant dysregulation in expression while *Myl6b* was the only disomic gene found to be significantly upregulated in the soleus muscles of Ts1Cje mice. Western blot analysis on three proteins of the myogenic regulatory factors (MRFs) family namely MyoD, Myf5 and Myg showed no significant difference in expression in both muscles. Following the targeted study, transcriptomic profiling of the soleus and EDL muscles of the Ts1Cje and wild-type disomic mice using microarray was performed. Results showed a total of 166

coding DEGs found in soleus muscles with 37 of them located on MMU16 and a total of 262 coding differentially expressed genes (DEGs) found in EDL muscles with 13 of them located on MMU16 (p-value ≤ 0.05, absolute fold change (abs FC) ≥ 1.5). Functional annotation clustering of these DEGs showed 5 significant functional clusters for soleus (signal transduction; development of reproductive system; nucleic acid biosynthesis; protein modification and metabolism and regulation of gene expression) and 3 significant functional clusters for EDL (neuron and cell development; protein modification and metabolic processes; and ion transport). Validation of nine selected genes which were found to be differentially expressed in both soleus muscles and EDL muscles was performed using RT-qPCR. The genes were *Cdk13, Mansc1, Ifnar1, Ifnar2, Donson, Dyrk1a, Runx1, Sod1,* and *Tmem50b* with the later 7 the trisomic genes. The analysis showed that all genes were upregulated in Ts1Cje soleus muscles by ≥ 1.5 fold while only *Don, Ifnar2* and *Tmem50b* were upregulated in Ts1Cje EDL muscles by ≥ 1.5 fold. Western blot analysis of two of the trisomic genes at the protein level showed a downregulation of Ifnar1 in Ts1Cje soleus muscles and downregulation of Ifnar2 in both soleus and EDL muscles of Ts1Cje mice as oppose to being upregulated in microarray and RT-qPCR analysis. Collectively, these findings showed an overexpression of the trisomic genes in Ts1Cje skeletal muscles, validating the hypothesis that the dysregulation of the genes caused by the triplicated region of MMU16 leads to muscle weakness in Ts1Cje. However, further investigation on the role of these genes and the protein expression levels may provide further insight on the underlying mechanism responsible for muscle weakness in Ts1Cje

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

ANALISIS TRANSCRIPTOME DAN PROTEIN OTOT RANGKA DARI MODEL MENCIT SINDROM DOWN Ts1Cje

Oleh

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Sindrom down (DS), adalah disebabkan oleh penambahan kromosom 21 manusia (HSA21). Sindrom meliputi fenotip klinikal seperti kecacatan intelek, ciri-ciri struktur muka yang berbeza, demensia permulaan muda, sistem imun yang lemah dan hipotonia. Pengurangan tonus otot serta fungsi motor dalam individu DS berlaku sepanjang tumberasan. Ini melibatkan kelemahan otot, pegenduran sendi yang melampau, kelewatan dalam kawalan postur dan kestidakeimbangan badan. Penyebab asas hipotonia bagi individu DS masih tidak diketahui dan kajian fisiopatologi dan pencirian di peringkat molecular mengenai hipotonia bagi DS adalah terhad. Ts1Cje merupakan model mencit DS yang mengandungi trisomi separa bahagian kromosom MMU16. Kromosom ini mempunyai jujukan terpelihara yang senteni dengan HSA21. Model mencit ini mempunyai ciri-ciri patologi deperti kelemahan otot yang sering dikaitkan dengan individu DS. Hipotesis kajian ini menyatakan bahawa ekspresi gen dari segmen trisomi MMU16 adalah lebih tinggi dalam otot rangka mencit Ts1Cje berbanding dengan mencit biasa lantas mengganggu jaringan molekul yang menyebabkan kelemahan otot mencit Ts1Cje. Untuk membuktikan hipotesis ini, kajian ini dibahagikan kepada dua bahagian iaitu kajian gen dan protein yang terlibat dalam pertumbuhan otot rangka terpilih berserta analisis trankriptom untuk mengenalpasti gen yang berkaitan dengan kelemahan otot dalam mencit Ts1Cje. Kajian ini menggunakan otot rangka "soleus" dan "extensor digitorum longus" (EDL) dari mencit Ts1Cje and mencit normal. Keputusan dari analisis transkripsi songsang - tindak balas berantai polymerase secara kuantitatif pada masa nyata (RT-qPCR) atas sembilan gen terpilih (*Lamc1, Leprel1, Myl6b, Msn, Pgm5, Tmod1, Istn1, Synj1* dan *Rcan1*) menunjukkan peningkatan ekspresi gen trisomi *Istn1, Synj1* dan *Rcan1* dalam otot rangka mencit Ts1Cje. Analisis gen disomi (*Lamc1, Leprel1, Msn, Pgm5* dan *Tmod1*) tidak menunjukkan sebarang perbezaan tahap ekspresi ketara kecuali *Myl6b* yang menunjukkan peningkatan secara ketara di otot soleus mencit Ts1Cje. Keputusan pedapan Western atas tiga protein daripada keluarga "myogenic regulatory factors (MRFs)" iaitu MyoD, Myf5 dan Myg menunjukkan tiada perbezaan ekspresi di kedua-dua otot rangka mencit Ts1Cje. Selepas kajian atas gen dan protein terpilih, analisis transkriptom soleus dan EDL dikaji dengan menggunakan teknologi cip tata susunan jujukan DNA mikro. Keputusan menunjukkan sebanyak 166 gen ekspresi

pembezaan (DEGs) di soleus dengan 37 gen daripada senarai DEGs merupakan gen trisomi MMU16. Sebanyak 262 DEGs diperolehi daripada analisis otot di EDL di mana 13 daripada DEGs tersebut merupakan gen trisomi MMU16 (berdasarkan kriteria nilai $p \leq 0.05$, perubahan kali ganda mutlak (abs FC) ≥ 1.5). Analisis pengelompokan anotasi berfungsi mengenal pasti 5 kelompok berfungsi yang ketara untuk soleus dan 3 kelompok berfungsi yang ketara untuk EDL. Pengesahan sembilan gen terpilih yang didapati mempunyai ekspresi pembezaan di soleus dan EDL mencit Ts1Cje dijalankan dengan RT-qPCR. Ini termasuklah *Cdk13, Mansc1, Ifnar1, Ifnar2, Donson, Dyrk1a, Runx1, Sod1,* dan *Tmem50b* di mana tujuh gen terakhir merupai gen trisomi MMU16. Analisis menunjukkan penningkatan tahap ekspresi (≥ 1.5 fold) semua gen di otot soleus mencit Ts1Cje dan hanya gen *Don, Ifnar2* dan *Tmem50b* menunjukkan peningkatan tahap ekspresi (≥ 1.5 fold) di otot EDL mencit Ts1Cje. Keputusan pedapan Western keatas dua protein gen trisomi menunjukkan pengurangan tahap ekspresi Ifnar1 di otot soleus mencit Ts1Cje dan Ifnar2 di kedua-dua otot soleus dan EDL mencit Ts1Cje. Keputusan ini bertentangan dengan keputusan berdasarkan teknik cip tata susunan jujukan DNA mikrodan RT-qPCR yang menunjukkan peningkatan ekspresi gen *Ifnar1* dan *Ifnar2*. Kesimpulannya, keputusan kajian ini menunjukkan bahawa gen trisomi didapati mengalami peningkatan tahap ekspresi lalu membuktikan hipotesis kajian yang menyatakan perubahan tahap ekspresi gen trisomi MMU16 menyebabkan kelemahan otot mencit Ts1Cje. Walau bagaimanapun, kajian lanjut perlu dilaksanakan ke atas peranan gen dan tahap ekspresi protein terlibat untuk mendalami pengetahuan tentang kelemahan otot mencit Ts1Cje secara khususnya, dan hipotonia pada individu DS secara amnya.

ACKNOWLEDGEMENTS

The decision to embark on this postgraduate journey was not an easy one to make. A decision to leave a job which I enjoyed to become a student once again took a lot of deliberation with endless lists of pros and cons although postgraduate studies had been in my plans after degree. Needless to say, I do not regret my decision and all this would not have been possible if not for the invaluable people who have made this journey a memorable one despite the ups and downs. Thus, I would like to express my deepest gratitude to all who have impacted my life in one way or another throughout this time.

Firstly I would like to thank my supervisor, Dr. Michael Ling for the continuous support throughout my Masters study. He never failed to encourage, guide and support me from the beginning to the completion of this thesis. I have gained much knowledge from his experience especially in the molecular field. His enthusiasm and countless scientific ideas is something I look up to. His generosity, kindness and jolly spirit made him a very approachable and caring supervisor. This also meant that I occasionally bother him a lot but he has always been patient and ready to give constructive feedback that is crucial for this achievement. He has a knack for working with each person differently thus allowing me enough freedom and independence while still keeping me on track. Thank you for all the motivational sessions and sharing your experiences, not just for scientific advices but also social and financial support. Thank you for being a role model as both a scientific researcher as well as a superior, creating a well balance environment to work and learn. I am truly grateful for the opportunity you have given me. You have been a great supervisor and I cannot imagine my Masters journey with a better supervisor.

I would also like express my sincere gratitude to my co-supervisor, Professor Dr. Rozita Rosli for her valuable guidance, endless support and constructive comments throughout. Her experience has been crucial in shaping my research and success of this thesis. Thank you for your time in sharing your input and advice on my work leading to the completion of my thesis. I also extend my appreciation to Associate Professor Dr Cheah Pike See for giving me constructive guidance and moral support throughout my journey. Her enthusiasm and creative ideas in anatomy has helped improved my knowledge and reduced the fear of it as it has never been my best subject. Thank you for always keeping me on track with constant reminders and datelines. Without them, my work would have not been as efficient.

The completion of my work would not be possible also if not for my colleagues. I would like to extend my sincere thanks to fellow NeuroBiology and Genetics Group (NBGG) members. A big thank you for occasionally sharing my experimental load, the scientific discussion, motivation, ideas, support, 'makan' sessions and friendship. I will cherish the memories and remember the moments of 'covering' for each other. It has been a pleasure working with all of you and I know our friendship will not end here. Cheers to being the best group in the lab. A special thanks to the seniors Angeline, Kai Leng, Han Chung and Usman who have guided me since the beginning and showing me the tricks of the trade. Thank you for your patience in teaching and helping me.

I would also like to dedicate my deepest gratitude to lecturers, staff and lab-mates from Genetic & Regenerative Medicine Research Centre (GRMRC) for their friendly assistance and support. The laboratory and this research would not have run smoothly without them. I could not have anticipated the fun we would have together. Thanks for making the lab a happening place, getting through the happy times and hardships together. Though the tears, joy, laughter and just a lot of fun. I enjoyed the random gossip sessions, long lunch breaks in the pantry, discussions or sometimes just sharing our complains on how things are just not working out but eventually get through them anyway. Needless to say the food, food and food which brings about legacy of everyone looking prosperous once joining the lab.

I also would like to take this opportunity to express my heartfelt thanks to my family members: to both my parents and brother for supporting me spiritually and emotionally throughout this journey. Thank you for your patience and understanding despite not knowing what I do most of the time. I will never forget your never-ending encouragement, motivation and supporting me in this path I have chosen. Thank you for sharing my ups and downs faced during this times.

Thank you to my friends outside of the lab and science world. The encouragement and support has made me happier and helped me forget about the problems and struggles during the hard times. Thank you for the random hang outs and catch up sessions despite us being geographically far from each other. Your constant questions of when will I finish has help remind me to stay on track and keep pushing to complete this thesis.

Lastly, I would like to acknowledge the Ministry of Education (MOE) for funding the MSc tuition fees under the MyBrain 15 scholarship programme and also to Universiti Putra Malaysia (UPM) for the financial assistance provided under the Special Graduate Research Assistant (SGRA) scheme. I am also thankful for the funding of this research project by Ministry of Higher Education, Malaysia.

The completion of this research and thesis would not be possible without every single one of you who have been a part of this experience. All glory to God for the completion of this journey. This would of course, be impossible without Him.

I certify that a Thesis Examination Committee has met on 15 May 2017 to conduct the final examination of Melody Leong Pui Yee on her thesis entitled "Transcriptomic and Protein Expression Analyses of Skeletal Muscles Isolated from Ts1cje Mouse Model for Down Syndrome" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

Members of the Thesis Examination Committee were as follows:

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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Name of Member of Supervisory Committee : Professor Dr. Rozita Rosli

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LIST OF ABBREVIATIONS

Abs FC	:	Absolute fold-change
App	:	Amyloid precursor protein
bp	:	Base pair
BSA	:	Bovine serum albumin
Cdk13	:	Cyclin-dependent kinase 13
cDNA	:	Complementary DNA
CNS	:	Central nervous system
DAVID	:	Database for annotation, visualisation and integrated discovery
DEGs	:	Differentially expressed genes
DEPC	:	Diethyl pyrocarbonate
DETs	:	Differentially expressed transcripts
dH_2O	:	Distilled water
DNA	:	Deoxyribonucleic Acid
DNACH11	:	Dynein axonemal heavy chain 11
Don	:	Donson
DS	:	Down syndrome
DSCR	:	Down Syndrome Critical Region
dt	:	Dinucleotides
Dyrk1a	:	Dual-specificity tyrosine-(y)-phosphorylation regulated kinase 1a
E	:	Embryonic
EDL	:	Extensor digitorum longus
GA	:	Gastrocnemius
GAPDH	:	Glyceraldehyde 3-phosphate dehydrogenase
gDNA	:	Genomic deoxyribonucleic acid
Grik1	:	Glutamate receptor, ionotropic, kainate 1
Hmbs	:	Hydroxymethylbilane synthase
HRM	:	High Resolution Melting
HSA21	:	*Homo sapien* autosome 21
Ifnar1	:	Interferon receptor 1
Ifnar2	:	Interferon receptor 2
Itsn1	:	Intersectin
Lamc1	:	Laminin, gamma-1
Leprel1	:	Leprecan-like protein-1
linc	:	Long non-coding
Mansc1	:	MANSC domain containing 1
MMU10	:	*Mus musculus* chromosome 10
MMU16	:	*Mus musculus* chromosome 16
MMU17	:	*Mus musculus* chromosome 17
MRF	:	Myogenic regulatory factors
mRNA	:	Messenger RNA
Mrp139	:	Multidrug-resistance-associated protein 139
Msn	:	Moesin

Mx1	:	Msh-like 1
Myf5	:	Myogenic factor 5
Myg	:	Myogenin
Myl	:	Myosin light chain
Myl6b	:	Myosin light chain 6B
MyoD	:	Myogenic differentiation protein
Neo	:	Neomycin
NTC	:	Non-template control
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
Pgk1	:	Phosphoglycerate kinase 1
Pgm5	:	Phosphoglucomutase-5
Psmb2	:	Proteasome subunit beta type-2
Rcan1	:	Regulator of calcineurin-1
REF	:	Reference
RIN	:	RNA integrity number
RIPA	:	Radioimmunoprecipitation assay
RNA	:	Ribonucleic acid
RT	:	Reverse transcription
Runx1	:	Runt-related transcription factor 1
SDS	:	Sodium dodecyl sulfate
SEM	:	Standard error mean
SNPs	:	Single nucleotide polymorphisms
Sod1	:	Superoxide dismutase 1
Synj1	:	Synaptojanin-1
TA	:	Tibialis anterior
TAE	:	Tris-acetate-EDTA
TBE	:	Tris-borate-EDTA
TIAM1	:	T-cell lymphoma invasion and metastasis 1
Tmem50b	:	Transmembrane protein 50B
Tmod1	:	Tropomodulin-1
Tris-HCl	:	Tris-hydrochloride
WT	:	Wild-type
Znf295	:	Zinc finger protein 295

CHAPTER 1

INTRODUCTION

1.1 Overall Study

Down syndrome (DS) is a genetic disorder caused by trisomy of human chromosome 21 (*Homo sapien* autosome 21 - HSA21). DS is the most common genetic form of intellectual disability and is characterised by a complex set of pathologies and clinical phenotypes. Some of the phenotypes associated with DS individuals are intellectual disability, characteristic set of facial features, dementia, weak immune system and hypotonia (Antonarakis et al., 2004; Wiseman et al., 2009). There are more than 80 clinical manifestations reported in DS individuals (Epstein et al., 1991). However, not all the characteristics are present in every DS individual. Intellectual disability, characteristic facial dysmorphology, hypocellular brain and hypotonia are some of the characteristics seen in all DS individuals (Antonarakis et al., 2004; Hassold et al., 2007; Wiseman et al., 2009).

Hypotonia which is muscle weakness is one of the phenotypes present in DS individuals. Studies have shown that individuals with intellectual disabilities generally show motor functions deficits throughout development (Palisano et al., 2001). Intellectual disability in DS has been widely characterised. To date, only limited studies on the pathophysiological and molecular characterisation of hypotonia in DS could be found. The causative mechanisms of muscle weakness in DS individuals remains unknown. This study intends to determine the dysregulated pathways and differentially expressed transcripts (DETs) caused by altered gene dosage of skeletal muscles in the wild type and DS mouse model.

Mouse is an invaluable model organism for the study to understand the genomic landscape that results in diseases in humans. This is due to its genetic and physiological similarities to humans. Orthologs of HSA21 genes are located on 3 mouse chormosomes (MMU) namely the telomeric region of MMU16, internal segments of MMU17 and MMU10 (Gardiner, 2004). The DS mouse model used in this study is the Ts1Cje which contains a segmental triplication of MMU16, from superoxide dismutase 1 (*Sod1*) (non-functional) to msh-like 1 (*Mx1*) (Antonarakis et al., 2004; Richtsmeier et al., 2000; Sago et al., 1998).

Ts1Cje exhibits several clinical phenotypes associated with DS individuals, such as enlarged brain ventricle, decreased neurogenesis, learning and behavioural deficits (Belichenko et al., 2007; Ishihara et al., 2010; Pennington et al., 2003; Reeves et al., 1995; Sago et al., 2000; Siarey et al., 2005) as well as muscle weakness and poor muscle coordination (Bala et al., 2016). Studies have demonstrated that the skeletal muscle strength of Ts1Cje is lower than average. Thus, Ts1Cje will serve as an ideal alternative model to define the genetic landscape, metabolic profiles and functional aspect of skeletal muscle leading to muscle weakness.

To date, there has not been any literature related to muscle weakness in Ts1Cje mice. Thus, this study aims to determine the underlying mechanisms of muscle weakness by studying the transcriptome of the Ts1Cje skeletal muscles. There are two types of skeletal muscle fibres, namely the slow twitch (Type I) and fast twitch (Type II) fibres (Boron and Boulpaep, 2011). The Type I muscle used in this study is soleus and Type II muscle is extensor digitorum longus (EDL).

1.2 Problem Statement

DS is the most common genetic form of intellectual disability with the estimated incidence of Down syndrome worldwide of 1 in 1,000 to 1 in 1,100 live births (World Health Organization, 2016). In Malaysia, the incidence of Down syndrome is approximately 1 in 800 live births (Ismail et al., 2009). Down syndrome has been linked with learning disabilities, characteristic facial dysmorphology, hypocellular brain and hypotonia (Antonarakis et al., 2004; Hassold et al., 1996).

Much research on Down syndrome has been focused on learning disabilities and hypocellularity of the brain. However, the cause of hypotonia and how trisomy of HSA21 is linked to hypotonia remains unknown. There is limited literature reporting on the functional characterisation, molecular mechanism and metabolic alterations underlying muscle weakness in Down syndrome individuals. Motor functions are an integral part of the human life and is necessary for even the most basic parts of our daily chores. The presence of hypotonia reduces the quality of life of the Down syndrome individuals limiting their mobility and ability to carry out basic day to day tasks is limited.

In general, DS children learn to walk, grasp objects and many other fundamental skills but their movements lack in precision, appear poorly coordinated and less efficient than the movements of normally developing children (Henderson et al., 1981). When comparing to normal individuals, both children and adults with DS are slower and more variable in reaction time task, clumsy and have poor movement coordination (Anson 1992). Musculoskeletal conditions have been shown to cause more functional limitations in the adult population as compared to other group of disorders which is a major cause of years lived with disability in all continents and economies (Woolf and Pfleger, 2003). They tend to lose their independence and instead having to depend on carers which in turn affects their education and general socio-economic status (Cunningham, 1996). This poses a financial impact to the families.

The economic burden of health care costs in the US for infants to 4 years of age with DS were found to be 12 to 13 fold higher in average and median medical costs compared with children of the same age without DS (Boulet et al., 2008). Another study in Australia showed that the total mean annual cost of medical care was $4,209 across age groups with a median cost of $1,701 (Geelhoed et al., 2011) where the major costs were hospitalisation and ‘other health costs’, comprising costs for therapy and respite care. Besides direct financial impact to the family, DS individuals require special care and attention for their livelihood. Special healthcare and welfare centres are dedicated to

improve the quality of life of the DS individuals which brings about a financial impact to the economy. A study in China showed the economic burden for the family and societal to be US$47,172 and US$54,871 respectively (Chen et al., 2008). Thus, understanding how having DS leads to muscle weakness is important.

Human samples, in particular skeletal muscle samples, are difficult to obtain for research. Collection of skeletal muscle samples are very invasive as they usually involve biopsies or surgeries leading to various ethical issues. Thus, an alternative animal model such as Ts1Cje mice will be a useful substitute to understand the role of gene dosage in hypotonia in Down syndrome.

1.3 Hypothesis

This study hypothesises that differential gene expression due to the partial triplication of MMU16 genes causes an alteration of gene dosage, disrupting the molecular pathways involved in the development and function of the skeletal muscles and therefore leading to muscle weakness.

1.4 Objectives

General Objective

This study aims to determine the candidate genes and disrupted molecular pathways due to the gene dosage effect in Ts1Cje skeletal muscles that are responsible for muscle weakness.

Specific Objectives

1. To evaluate the expression of selected genes associated with skeletal muscle development and function.
2. To identify the differentially expressed transcripts in adult skeletal muscles of wild type and Ts1Cje mouse.
3. To determine disrupted molecular pathways responsible for hypotonia in Ts1Cje mouse.
4. To validate the selected differentially regulated transcripts and proteins in the skeletal muscles of wild type and Ts1Cje mouse.

1.5 Significance of Study

The fundamental knowledge of genotype to phenotype (muscle weakness) correlations in Ts1Cje mice model for DS will enhance the current understanding of mechanisms underlying muscle weakness. The effort to elucidate the molecular mechanisms underlying muscle weakness in Ts1Cje mouse model will serve as fundamental knowledge to understand hypotonia in DS individuals. The results of the study will eventually be used to formulate targeted investigations in human DS skeletal muscle in the future as well as development of more effective therapy for muscle weakness in DS individuals. This will potentially lead to improvements in the quality of life of DS individuals, later impacting the social and economy of the country positively.

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