The role of forkhead box A2 in skeletal muscle

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2019

Phua, W. W. T. (2019). The role of forkhead box A2 in skeletal muscle. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/106796>

<https://doi.org/10.32657/10220/49672>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

The Role of Forkhead Box A2 in Skeletal Muscle

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Interdisciplinary Graduate School

Institute for Health Technologies (HealthTech NTU)

2019

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A thesis submitted to the Nanyang Technological University in partial

fulfillment

of the requirement for the degree of

Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

18th January 2019

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A handwritten signature in black ink, appearing to read 'Wendy', written over a horizontal dotted line.

.....

Wendy Phua

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it of sufficient grammatical clarity to be examined. To the best of my knowledge, the thesis is free of plagiarism and the research and writing are those of the candidate's except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Authorship Attribution Statement

This thesis contains material from 1 paper published in the following peer-reviewed journal where I was the first and/or corresponding author.

Chapter 1 is published as WWT. Phua, Wong MXY, Liao Z and Tan NS, An aPPARent Functional Consequence in Skeletal Muscle Physiology via Peroxisome Proliferator-Activated Receptors. *International Journal of Molecular Sciences* 19(5), 1425 (2018). <https://doi.org/10.3390/ijms19051425>

The contributions of the co-authors are as follows:

- A/Prof Tan provided the review direction and edited the manuscript drafts.
- I prepared the manuscript drafts. The manuscript was revised by myself and A/Prof Tan.
- I co-designed the content of the review article with A/Prof Tan and performed all the literature writing in the School of Biological Sciences.
- All figures presented in the review was prepared by myself.
- Wong Melissa Xin Yu and Liao Zehuan assisted in the collection of literature information and assisted in the content layout of the manuscript.

18 January 2019

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Date

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Acknowledgment

You only live once.

While this aphorism has taken the world over in the early 21st century, it became an idealism of mine that calls for excitement to embrace fears and every associated inherent risk. I owe my gratitude to these people and I am taking this opportunity to extend my heartfelt thanks.

First and foremost, I owe my profound gratitude and sincere thanks to Associate Professor Tan Nguan Soon Andrew, my supervisor, for his scientific insights, his guidance and his extension of helping hands at various times when I faltered. From time to time, his insightful comments and constructive criticisms have led me to become a more efficient person and a better thinker. I am very grateful for the opportunity and the experience of being a part of his lab.

I owe my deep gratitude and heartfelt thanks to Professor Walter Wahli, my co-supervisor, for his magnanimous support and warmth throughout the course of my research. I am grateful for his advice, encouragement and guidance from the early stage of my work. His fiery passion in science has kept me constantly inspired my growth as an individual I would like to be.

I sincerely thank the members in my thesis advisory committee, Associate Professor Melvin Leow and Assistant Professor Yusuf Ali for their insightful comments and valuable perspectives on the knowledge gaps. I also wish to take this opportunity to thank my examiners for their time for this thesis.

I express my sincere appreciation to Professor Professor Bo Liedberg and his IGS team, especially Ms. Suriani Rabu and Ms. Lena Tay, for their encouragements and administrative supports throughout the course.

I thank Dr. Ravi Kambadur for his nomination and mentorship during the early course of my research, and his team in A*STAR SICS, especially Dr. Piyush

Khandelia, Dr. Shi Yu, Dr. Gavian Lua, and Mr. Joshur Ang for their genuine advice and support during difficult times.

I am thankful for Mr. Marcus Liang for the 7 years of fun, like-mindedness habits and reliance. I am grateful for his endurance, patience and company over difficult times. With no doubts, our leap from comfort zones will be highly remembered which will continue to grow us as individuals.

I profess my sincere gratitude to the past and present lab members, Mr. Jonathan Wee, Mr. Mark Tan, Mr. Yip Yun Sheng, Mr. Low Zun Siong, Miss Jeannie Lee, Dr. Cheng Hong Sheng, Dr. Maegan Lim, Dr. Eddie Tan, and Dr. Jeremy Chan whom shared their queer interest in Pokemon Go, precious company and encouragements. Hereby, I extend my depth of gratitude again to Mr. Jonathan Wee for his unwavering trust and patience, his constant injection of positivity and honesty, as well as our strong friendship fostered through the highs and lows. I express my sense of appreciation to Mr. Tan Ming Jie, Dr. Li Liang, Dr. Zhu Pengcheng, Dr. Tan Zhen Wei, Dr. Terri Phua and Dr. Sng Ming Keat for their advice and guidance along the way. I must also thank Ms. Rachel Tee for her timely assistance throughout the course of my doctorate. Notable mentions and my heartfelt thanks must also extend to Miss Erica Teo, and all FYP students -- Mr. Ooi Chin Kiat, Mr. Joseph Lim, Mr Kong Ze Qing, Mr. Toh Jun Hong, Mr. Ridley Lim, Mr. Benjamin Henategala, Mr. Soong Jen Chi, Mr. William Tan, and Mr. Ivan Hew for their presence, their friendships and their constant share of laughter that made late night experiments more bearable.

I thank Nanyang Technology University for providing the life-changing opportunity 9 years ago when I yearned for a switch in the course of study from chemistry to life science. It has been one of my best decisions ever since. I am also thankful for the subsequent scholarship awarded for the pursuit of my Ph.D.

Finally, this thesis would not have been possible without the love of family. My family has been unconditionally supportive of my decisions, despite my selfishness in pursuit of knowledge. It is their love, their understanding and their

patience that sheltered and gave strength to me through the psychological turmoil of the Ph.D. Special honourable mention to little Andre and Asher, whom bring great fun and relief since their arrival, ironically through their cries and mischiefs.

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LIST OF ABBREVIATIONS

Acyl-CoA	Acyl-coenzyme A
AF1	Activation function 1
AF2	Activation function 1
ANGPTL4	Angiopoietin-like 4
ARE	Androgen response element
BCL6	B-cell lymphoma 6 protein
BSA	Bovine serum albumin
cAMP	cAMP response element-binding protein
CD36	cluster of differentiation 36
ChIP	Chromatin immunoprecipitation
Cort	Hydrocortisone
CPT1A	Carnitine palmitoyltransferase I A
CREB	AMP response element-binding protein
CXCL10	C-X-C motif chemokine 10
DBD	DNA binding domain
Dex	Dexamethasone
DLK1	Delta Like Non-Canonical Notch Ligand 1
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DR	Direct repeat
FABP3	Fatty-acid-binding protein 3
FABP5	Fatty-acid-binding protein 5
FBS	Fetal bovine serum

FFA	Free fatty acid
FHD	Forkhead domain
<i>fkh</i>	<i>Forkhead</i>
FoxA2	Forkhead Box A2
Gas	Gastrocnemius
GLUT4	glucose transporter
GO	Gene ontology
GR	Glucocorticoid receptor
GW	GW501516
h	hour
HH	Hyperinsulinaemic hypoglycemia
HK2	Hexokinase 2
HNF3B	Hepatocyte nuclear factor 3B
IGFBP5	Insulin like growth factor binding protein 5
IL6	Interleukin 6
IPA	Ingenuity pathway analysis
IRS1	Insulin receptor substrate 1
I κ B	Inhibitor of kappa B
LBD	Ligand binding domain
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
LXR	Liver X receptor
MCP1	Monocyte chemoattractant protein 1,
MMP9	Matrix Metalloproteinase 9

MODY	Maturity diabetes of the young
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
NCoR	Nuclear receptor co-repressor 1
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
OA	Oleic acid
PA	Palmitic acid
Pax3	Paired box protein 3
Pax7	Paired box protein 7
PDK	Pyruvate dehydrogenase kinase
PGC-1 β	Peroxisome proliferator-activated receptor gamma coactivator 1-beta
PI3K	Phosphatidylinositol-3-kinases
PPAR	Peroxisome proliferator-activated receptor
PPRE	PPAR response element
qPCR	Real-time polymerase chain reaction
Quad	Quadricep
RNA	Ribonucleic acid
RXR	Retinoid X receptor
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SMRT	Silencing mediator for retinoid and thyroid receptors
Sol	Soleus
T-tubules	Transverse tubules

T2DM	Type 2 diabetes mellitus
TA	Tibialis anterior
TCF3B	Transcription factor 3B
Th2	Type 2 helper T cell
Tnfa	Tumor necrosis factor alpha
TOR	Target of rapamycin
TZD	Thiazolidinediones
WHO	World Health Organization

ABSTRACT

Skeletal muscle, besides its physical function in locomotion, is also a massive metabolic organ which plays an essential role in whole-body energy homeostasis. Skeletal muscle is responsible for over 80% of peripheral glucose uptake and a major consumer of fatty acids. Forkhead box A2 (Foxa2), a member of the forkhead box class of DNA-binding transcription factor, is known to regulate genes involved in post-natal lipid and glucose homeostasis. Majority of current literature on Foxa2 focuses on metabolic organs, this include liver, pancreas, and adipose tissue. However, the expression and the functional role for Foxa2 in skeletal muscle is unknown. Using *in vitro* and *in vivo* experiments, we showed the presence of Foxa2 in skeletal muscle, and that ligand activation of peroxisome proliferator-activated receptors (PPARs) directly increased its expression. In contrast to the liver, skeletal muscle Foxa2 expression was refractory to hormonal regulation by insulin and glucocorticoids, indicating a tissue-specific regulation of Foxa2. Gene ontology and Ingenuity Pathway Analysis of known downstream targets of Foxa2 revealed pathophysiological roles of Foxa2 primarily in lipid metabolism and inflammation-associated injury. The shRNA knockdown of Foxa2 in C2C12 myoblasts followed by focused PCR array showed significant upregulation, albeit marginally, of genes involved in lipid transport and glucose metabolism. Notably, the mRNA levels of prototypical pro-inflammatory genes such as Il6 and Tnf α showed a steep increase. *In vivo*, the intramuscular injection of carrageenan, which mimics localized exercise-induced inflammation, lowered the expression of Foxa2. PPAR-mediated upregulation of Foxa2 mitigated the acute inflammation-

associated muscle damage. Taken together, we demonstrated that Foxa2 in skeletal muscle plays a cytoprotective role against aggravated inflammatory response.

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CHAPTER 1: LITERATURE REVIEW

1.1 Skeletal muscle

The human body consists over 650 named skeletal muscles that contribute to approximately 40% - 50% body weight of a healthy individual [1]. Skeletal muscle is one of the three types of muscles, the other being cardiac muscle and smooth muscle [1]. Unlike cardiac and smooth muscle which are innervated by the autonomic nervous system, skeletal muscle is innervated by the somatic nervous system which is under conscious control [2]. In mammals, the formation and growth of skeletal muscle occur over 3 phases, namely during embryonic development, neonatal growth and post-natal regeneration [3]. Skeletal muscle contributes significantly to multiple bodily functions, and it is well-recognized for their function in force generation for locomotion, respiration, postural support and heat production, all of which are fixed at birth [2]. Skeletal muscle can withstand massive and sudden changes, both mechanically and bioenergetically, and allows voluntary movement and posture maintenance through coordinated contraction and relaxation of the skeletal muscle [4]. During postnatal muscle growth, skeletal muscle can undergo cellular changes, increases in length, girth and myofiber numbers in response to the nutrient availability [5]. Besides its well-recognized mechanical capacity, skeletal muscle also contributes significantly in whole body metabolism, overall health, and quality of life [1]. From a metabolic perspective, skeletal muscle contributes to basal energy metabolism, serves as energy as well as heat production for the maintenance of core body temperature [2]. Robust muscle function is thus essential for the maintenance of overall health and quality of life [2].

1.2 Skeletal muscle structure

Each skeletal muscle is considered an organ of the muscular system [2]. As an organ, each muscle is surrounded by connective tissues, nerves, and blood vessels [6]. Skeletal muscles are made up of fascicle bundles which composed of elongated multinucleated syncytia, that can range from 1 mm to 40 mm in length [4]. Each muscle is surrounded and subdivided by layers of connective tissues (Figure 1), with the outermost layer that surrounds and separates the muscles known as the epimysium [7]. The epimysium is also the dense connective tissue that generally extends beyond the muscle tissue to form an attachment with the periosteum of bones or to the connective tissue or other muscle [7]. Portions of the epimysium project inward as well, which subdivide and compartmentalize muscle fibers into bundles [6]. Each muscle bundle, or better known as fascicle, is separated from each other by perimysium [7]. Muscle bundles can vary in size from 50 to 300 muscle fibers per bundle [6]. Within each fasciculus, individual muscle fiber, or myocyte, is surrounded by the endomysium [7]. These connective tissues provide mechanical support for the soft skeletal fibers and network structures for blood vessels, lymphatic vessels, and nerves [2]. In addition, each skeletal muscle fiber is coated by a layer of an extracellular matrix known as the basement membrane [8]. The basement membrane further consists of two layers: an internal basal lamina that directly linked to the sarcolemma, and a thin external fibrillar reticular lamina [8]. The basement membrane contains mostly glycosylated proteins and proteoglycans which maintains the muscle integrity and provide a scaffold during muscle regeneration [8].

Each muscle cell, or myocyte, is surrounded by sarcolemma which generally maintains the same function as the plasma membrane in other cell types [2].

Sarcolemma invaginates into the sarcoplasm to form transverse tubules (T-tubules) that allow rapid transmission of neuronal stimuli [9]. Each myofiber consists of highly organized cytoskeletons composed of myofibrils that run in parallel to each other [10]. Each myofibril consists of repeated organization of actin and myosin myofilaments known as sarcomeres [10]. Sarcomeres are the basic functional units of skeletal muscle that facilitate force generation through the sliding of myofilaments [10].

Figure 1.1 Schematic representation of skeletal muscle structure

A whole skeletal muscle is considered as an organ of the muscular system, each skeletal muscle is surrounded by layers of connective tissue, termed epimysium, perimysium and endomysium. Each myocyte contained chains of myofibrils which are composed of actin and myosin filaments organized in repeated units known as sarcomeres. The muscle tissues are surrounded by a network of blood capillaries and motor neurons to provide nutrients and signals. This image was reprinted and modified from Principles of Anatomy and Physiology, 9th edition, by G. Tortora and S. Reynolds (1999) John Wiley and Sons. Copyright 2018 by John Wiley and Sons.

1.3 Embryonic and post-natal development of skeletal muscle

Skeletal muscle originates from the paraxial mesoderm, which gives rise to somites [11]. Within somites, skeletal myogenesis is initiated and forms the first pre-myogenic progenitors and skeletal myoblasts [12]. The somite gives rise to the dermomyotome where muscle progenitor cells reside and signs of myogenesis first occur [13,14]. The dermomyotome gives rise to the myotome, where the first myocyte in the embryo is formed [12]. The embryonic myogenesis occurs in two phases (Figure 1.2), the primary myogenesis and the secondary myogenesis [15,16]. The primary phase gives rise to the primary myofibers, which derived from paired box homeodomain-3 transcription factor Pax3⁺ dermomyotomal progenitor cells, forms the early myotomes and limb muscles [17]. These primary myofibers also give rise to a fraction of satellite cells residing in post-natal skeletal muscle [17,18]. The secondary myogenesis occurs as a subset myogenic progenitors begins to express Pax7 but reduces Pax3 expression [12]. Using the primary fibers as scaffolds, these Pax7⁺/Pax3⁻ myogenic precursors promote and sustain the growth of fetal muscle through cell fusion [19]. Mature skeletal myofibers form through the fusion processes of myoblast-myoblast

and/or myoblast-myotube to produce multinucleated matured myotubes [20]. A subset of these Pax7⁺ progenitors cells will also form the pool of satellite cells [17,18].

Figure 1.2 The general stages of differentiation from somitic progenitors cells to skeletal muscles and satellite cells.

The myogenic stem cells, which originates from the somite, contribute towards embryonic myogenesis and satellite cells. The adult satellite cells eventually located on mature myofibers in the satellite cell niche. This image was reprinted and modified from Cha et. al (2017) Development 144: 2104-2122; DOI: 10.1242/dev.151035 Permission to use this copyrighted image is granted by RightsLink online copyright clearance center (See appendix 2).

Postnatal and adult skeletal muscle tissue, like any other tissue, is in a dynamic state with continuous turnover due to daily minor traumas and small lesions [3]. During the early postnatal life, satellite cells in skeletal muscle tissue are plentiful and they are accounted for nearly 30% of all nuclei identified underneath the basal lamina [21]. The satellite cells are uniquely located between the sarcolemma and basement membranes, especially at the ends of fibers where longitudinal growth in skeletal muscle takes place [22]. The relative abundance of satellite cells gradually declines within adult skeletal

muscles to 5% or less [23]. Adult skeletal muscle has regenerative capacity and subjected to a continuous and frequent turnover, as well as in response to prominent injury or recurrent traumas [24]. In general, postnatal regenerative myogenesis due to an injury of skeletal muscle can be divided into three main stages; degeneration, regeneration, and remodeling [3]. Postnatal myogenesis involves a highly synchronized process, where coordination between inflammation and regeneration is crucial following muscle damage [25]. During the early phase of muscle injury, the activation of inflammatory cells and satellite cells is regulated by the factors released by the injured muscle [26]. The degeneration event is accompanied with rapid fiber necrosis and an increased number of non-muscles mononucleated cells within the injured area [27]. During the regeneration phase, the activated satellite cells generate myoblasts which then differentiate and fuse [25]. The fusion of the myogenic cells may occur as myoblast-to-myotube in reparation of existing damaged fibers, or myoblast-to-myoblast for new myofiber formation [28]. In the remodeling stage of muscle regeneration, the site of muscle injury is surrounded and repaired through the formation of extracellular matrix which further promotes muscle development and arrangement of the cells within tissues [25].

The terminal differentiation of myocytes is governed by four basic helix-loop-helix (bHLH) transcription factors that are known as the myogenic regulatory factors (MRFs), whose coordinated expression drives muscle-specific gene expression [29]. Myogenic factor 5 (Myf5) and myoblast determination protein (MyoD) control timely myogenic progression in satellite cells and proliferation of skeletal muscle precursor cells during regeneration [29]. The self-renewal and myogenic differentiation of satellite cells in postnatal muscle regeneration occurs through the regulation of Pax7

and MyoD expression, in which a subset of satellite cells maintain Pax7 expression while decreasing MyoD expression [30]. Myogenin (MyoG) is critical for myogenic differentiation of committed precursor, and together with muscle-specific regulatory factor 4 (MRF4), they promote the myogenic differentiation program [31]. Myogenin is expressed temporally and spatially during cell cycle withdrawal of myoblasts, permitting the transition from proliferating myoblasts to differentiating myotubes, activates the expression of the muscle structural protein myosin heavy chain, and facilitating myotube fusion [32,33]. The collective actions of MRFs appears highly concerted and cumulative to induce downstream muscle and non-muscle specific genes and/or further auto-regulate its own level of expression [34].

Figure 1.3 Postnatal myogenesis in mammals.

Illustration of lineage progression of postnatal skeletal muscle myogenesis. Pax3/Pax7 is expressed in muscle stem/ progenitors and downregulate upon the commitment to the skeletal muscle lineage. At proliferative stages, precursor cells no longer express purely

satellite cell markers (Pax3 and Pax7) but express myogenic markers such as MyoD and form myoblasts. Myogenin, which is not indicated here, is required for muscle differentiation which promotes the fusion of differentiated myoblast to myoblast, myoblast to existing myotubes, or myotubes to myotubes. Myosin, a major component of the muscle contractile apparatus, is expressed in differentiated myocytes. A subset of activated Pax7⁺ satellite cells will return to quiescence. Figure adapted and modified from Sambasivan et al. (2007) *Seminars in Cell and Development Biology*, 870-882, DOI: 10.1016/j.semcdb.2007.09.013. This material is reproduced with permission from Elsevier provided by Copyright Clearance Centre (See Appendix 3).

1.4 Muscle fiber types

Superficially, skeletal muscles appear similarly alike at various anatomical locations. At the cellular level, distinctively, skeletal muscle is composed of an array of heterogeneous fibers with different functions and compositions [35]. The mammalian skeletal muscle can be classified into a spectrum by their contractile and metabolic properties, but broadly into two categories: type I fibers and II fibers [35]. Type I fibers are rich in mitochondria and have a higher insulin sensitivity and glucose transporter 4 (GLUT4) expression levels than the fast-twitch type II fibers [36]. The type I fibers are rich in myoglobin, surrounded by many capillaries, and contain relatively abundant intracellular lipid levels for oxidative metabolism [35]. These characteristics support long-duration contractile activities and provides continuous energy, such as postural stabilization, walking and long-distance running [37]. Type I fibres are thus also known as the slow-twitch fibres [38]. In contrast, the type II fibers are large fibers with vast glycogen reserves that support their role in glycolytic metabolism [39]. Type II fibers produce rapid contractions that are used for intense activities, but these fibers are easily fatigued. In mammals, type II muscle fibers can be further categorized into type IIa

(oxidative), type IIb (glycolytic), and type IIx (an intermediate type between IIa and IIb). In humans, type IIb fibers are not detectable [35]. The heterogeneity of skeletal muscle provides the framework of flexibility, from activities that require continuous low-intensity long-lasting contraction, to repeated submaximal contraction and to high-intensity short-duration contractions [35]. Furthermore, fiber type diversity may also reflect adaptation to whole-body metabolism [35]. Oxidative muscle fibers are more active than glycolytic fibers during post-prandial glucose uptake, while glycolytic fibers are more sensitive to energy respiration [40] and plays a role in modulating hepatic fatty acid β -oxidation through organ crosstalk [41]. Muscle fiber thickness is in general inversely related to its aerobic oxidative activity [35].

1.5 Skeletal muscle metabolism in health and disease

Skeletal muscle, as the largest metabolic organ, is heavily influenced by the dynamic status of glucose and fatty acids, and in turn, highly influential to the metabolic state of the body [2]. Following a meal, skeletal muscle accounts up to 80% of post-prandial glucose uptake via an insulin-dependent mechanism [42]. Due to the overall mass of skeletal muscle and its high responsiveness to insulin, excess glucose taken up from the bloodstream gets incorporated into muscle glycogen [43]. During exercise or other physical exertion, skeletal muscle utilizes large amount of glucose, through the uptake from bloodstream and muscle glycogen breakdown for energy production [44]. Although skeletal muscle can utilize fatty acids as a source of energy production, glucose deprivation during prolonged and intense activity can cause sudden fatigue [45]. Under fasting conditions, when plasma insulin concentration is low, muscle energy metabolism switches from glucose oxidation to fatty acid oxidation, exclusively leaving

glucose utilization for the brain [46]. Such ability of muscle metabolic switch between fatty acid and glucose oxidation depending on the body's nutrient availability has been referred to as metabolic flexibility [47].

Moreover, skeletal muscle serves as the principal reservoir for amino acids that maintain whole-body protein synthesis [48], as well as the maintenance of blood glucose during periods of starvations and prolonged absence of nutrient uptake [49]. Altogether, skeletal muscle demonstrates high plasticity to nutrient availability and diet, and thus the physiological skeletal metabolic health status has the potential to substantially impact whole body metabolism as well as physical function [48].

1.5.1 Obesity and type 2 diabetes

The World Health Organization (WHO) estimated that more than 650 million people were obese in 2016 and the prevalence of obesity has nearly tripled since 1975 [50]. The risks of adverse metabolic syndromes and related diseases such as insulin resistance, type 2 diabetes mellitus (T2DM), and coronary heart disease, increased steadily in obese and overweight. T2DM, of which obesity is the single best predictor of this disease, is characterized by chronic hyperglycemia due to a combination of insufficient insulin production from pancreatic β -cell and peripheral insulin resistance [51], and a largely preventable disease through healthy lifestyle [50]. The WHO estimated 422 million adults are living with diabetes in 2014, which comprises of both type 1 and type 2 diabetes [52]. Type 1 diabetes, unlike T2DM, is an auto-immune condition in which pancreatic cells are destroyed leading to the insufficient production of insulin [53]. global estimates for the prevalence of diabetes is predicted to increase to 642 million by 2040, and the prevalence of the condition has been regarded as a

global epidemic [54]. T2DM accounts for around 90% of the total cases of diabetes worldwide, while type 1 diabetes account for at 5-10% of the cases [52]. Moreover, the unprecedented aging population is a major contributor to the diabetes pandemic, of which older adults with sedentary lifestyle have the highest prevalence of diabetes across all age-groups [55].

Unlike in syndromes associated with aging where loss of muscle mass and strength exhibit obvious impact to the role of skeletal muscle, the deregulation of muscle metabolic function during the development of obesity and T2DM is less well appreciated [48]. The deregulation of insulin-stimulated glucose uptake by skeletal muscle hence plays a major role in the pathogenesis of insulin resistance and T2DM [56].

Insulin resistance is widely established to precede the development of the overt hyperglycemia [57]. In the presence of robust insulin compensatory response by the pancreas, glucose levels can remain relatively normal in individuals with insulin resistance. Prolonged impairment of glucose tolerance and insulin sensitivity in peripheral tissues however leads to the progressive decline of insulin secretion as a result of overworked pancreatic β -cells. When the pancreas can no longer compensate the body's demand for insulin, it manifests the development of hyperglycemia and eventually T2DM [57]. As the consequences of chronic, long-term overnutrition, insulin resistance and obesity usually manifested through complex and heterogeneous mechanisms that involve increased FFA uptake, secretion of pro-inflammatory cytokines and chronic low-grade inflammation [58]. Besides impaired glucose uptake, the increased myocellular lipid accumulation and mitochondrial dysfunction due to the

reduced skeletal muscle oxidative capacity have been considered as important contributors to the impairment of skeletal muscle function [59]. This impaired metabolic switch from fatty acid to glucose oxidation in skeletal muscle, also termed as impaired metabolic flexibility, thereby contributes to insulin resistance in skeletal muscle [60]. There is ample evidence showing that the elevated plasma free fatty acids and ectopic lipid accumulation as a result of obesity, induce insulin resistance through direct interference with insulin signaling and glucose utilization in skeletal muscle [59,61,62]. Several mechanisms of free fatty acids (FFA)-induced insulin resistance have been proposed, however, the exact cellular cause and its molecular mechanism of metabolic disturbance in skeletal muscle remained to be fully defined [63].

1.6 Cellular regulation of metabolism

The overall regulation of metabolism in mammals is tightly maintained in a sophisticated manner by the automatic nervous system through electrical signals and neuropeptides, and the endocrine system that secretes hormones [64]. At cellular level, diverse mechanisms are employed to modulate metabolic flux and regulate metabolism that conform to the needs of the cell [65]. One of the classical aspects of metabolic control in the biological system is the allosteric effects of small molecules on enzymes along metabolic pathways [66]. Allosteric regulation of enzymes triggered through the binding of small molecules leads to conformational changes and thereby enhances or inhibits the enzyme's activity. Another means of regulation involves various post-translational and enzymatic modifications such as acetylation, phosphorylation, and ubiquitination, that provide rapid cellular signaling and feedback mechanism of the cell's metabolic status [65]. The third mechanism is the transcriptional regulation, in

which the abundance of enzymes is controlled through messenger RNA (mRNA) transcription, post-transcriptional modification and translation [67]. Often, transcriptional regulation of metabolism exerts control over longer timescales and involves isozymes and transcription factors that allows tissue- and context specific responses [67]. Most metabolic regulations are subjected to the coordination of this mechanism with the ultimate goal to parse metabolites to match the requirements of individual cells [65].

1.7 Forkhead box transcription factors

The forkhead box (Fox) transcription factors constitute an evolutionarily conserved family of proteins that play a central role during embryonic development and adult tissue homeostasis [68]. The Fox genes are characterized by a highly conserved winged-helix DNA binding domain, which spans about 110 amino acids long, also referred to as the forkhead domain (FKD) [69]. The Fox gene was originally identified in *Drosophila melanogaster* whose mutations resulted in characteristic spiked head appearance, thus coining the term Forkhead [70]. Fox genes have been identified in eukaryotes from yeast [71], nematodes [72], zebrafish [73] to mice and humans [74]. The Fox family consists of 50 members in the human genome, and 44 members in the mouse genome [74]. Thus far, the Fox proteins are divided into 19 subclasses from FOXA to FOXS, grouped according to the structure and degree of homology of their DNA binding domain (Figure 1.4) [69,75]. In vitro studies on the specificity of the FKD identified a generic consensus seven base pairs DNA consensus sequence 5'-(G/A)(T/C)(A/C)AA(C/T)A-3' recognized by the Fox transcription factors [75]. Homozygous deletion of a Fox gene, in many cases, leads to embryonic lethality [68]. In humans, mutations in Fox genes have been linked to several diseases ranging from

infertility, language and/or speech disorder and immunological defects [75]. Moreover, many Fox proteins have already been suggested to act as either tumor suppressors or oncogenes upon dysregulation, due to their role in transcriptional signaling and in the regulation of tissue homeostasis [75]. The transcriptional regulation by Fox transcription factors, and their implication in physiology and pathology, thus bears witness to their complexity and importance not only during development but also in the adult life [68]. Consequently, unraveling the specificities of Fox members in details promotes further understanding of Fox members for therapeutic use [69].

Figure 1.4 Alignment of forkhead box (fox) genes in mice.

Protein sequence alignment of the forkhead (FKH) domain all 44 forkhead box family members in *Mus musculus*. The FKH domain is the only consistently conserved portion of the protein across all members of the Fox family, whereas there are limited similarities in other regions among the Fox subfamilies. The first 3 protein alignment showed the similarity of the Foxa members. Figure adapted from Hannenhalli et al. (2009) *Nature Reviews Genetics*, 233-240, DOI: 10.1038/nrg2523. This material is

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1.8 Forkhead box A2

Forkhead box A2, previously known as HNF3B (hepatocyte nuclear factor 3B) or TCF3B (transcription factor 3B), belongs to the FOXA subfamily of Fox transcription factor and has been shown to be critical for embryonic development and post-natal metabolic homeostasis [76]. The mammalian FOXA subfamily, which comprises of Foxa1, Foxa2, and Foxa3, is the most closely related to the *Drosophila* protein fork head (*fkh*) and was initially discovered from rat liver nuclear extracts [77]. The FOXA proteins sequences share 95% similarity in their FHD and are flanked by nuclear localization sequences. Beyond their FHD, the members share weak homology, and with Foxa3 being less closely related than Foxa1 and Foxa2 in general [76,78]. Functional analysis done by several studies on Foxa2 revealed that the regions at both C and N terminus act as a transcriptional activator and its ability to regulate the transcription of its target genes [79,80]. The FOXA proteins are known as the pioneer factors during embryogenesis which displace linker histones, unwind compacted chromatin and facilitate the binding of other transcription factors [81]. The *Foxa2* gene is first activated among the FOXA family during embryogenesis, with Foxa2 expression first detected at day 6.5 (E6.5) in mice embryo [76]. In mice, Foxa2 exerts critical role in the development of the endoderm and midline structures, including the notochord, somites and axial mesoderm [82,83]. Mice with homozygous *Foxa2* null mutation exhibit early embryonic lethality at E9-11 due to severe defects in embryonic pattern of the primary body axes [68].

1.8.1 Role of FOXA2 in metabolism

Foxa2 orchestrates post-natal transcriptional regulation of glucose and lipid homeostasis in metabolically active tissues such as liver, pancreatic β cells and adipocytes [84-90]. The transcriptional regulation of Foxa2 is sensitive to nutrition status and hormonal regulation such as glucocorticoid and insulin [90-92]. During fasting, Foxa2 promotes transcriptional control of hepatic fatty acid oxidation via co-activation with PPAR γ coactivator 1-beta (PGC-1 β), as well as ketogenesis [89,92]. In postprandial state, Foxa2 is phosphorylated at threonine residue 156 through insulin-mediated PI3K/AKT signaling, which lead to Foxa2 nuclear exclusion and inhibition of its target genes [91,93]. Constitutively active Foxa2 expression in hepatocytes led to an increased expression of genes encoding for fatty acid oxidation and glycolysis, resulting to a decreased in hepatic triglyceride content, reduced glucose production, and normalized plasma glucose level [92]. Conversely, mice with chronic hyperinsulinemia resulted in the inactivation of Foxa2 in the liver, thereby manifesting lipid accumulation and insulin resistance [92]. In mice with specific Foxa2 deletion in pancreatic β cells, these mice are unable to secrete insulin as a response to elevated blood glucose concentration, yet inappropriately secrete in response to increased amino acids levels, thereby resulting in hypoglycemia and relative hyperinsulinemia [85,87]. Interestingly, while Foxa2 ablated mice are not viable, mice with haploinsufficiency in Foxa2 exhibit normal lifespan and fertility but developed increased adiposity upon high-fat diet. [88]. In humans, mutations in FOXA2 and/or at its respective cis-regulatory consensus binding sites have been linked to diabetes susceptibility [94]. FOXA2 in humans has also been linked to congenital hyperinsulinaemic hypoglycemia (HH), the most common cause of severe and persistent hypoglycemia in newborns and children, due to dysregulated insulin secretion that persists in the presence of low blood glucose levels

[95]. Undiagnosed and/or untreated HH may lead to irreversible hypoglycemic brain damage [96]. Furthermore, hyperinsulinemia is also a common feature of obesity, insulin resistance and during the development of T2DM [97]. The inhibition of Foxa2 regulation by insulin has thus been suggested to cause the metabolic balance of fatty acid oxidation and triglyceride degradation, leading to insulin resistance [92].

1.8.2 Cross-talk interactions between Foxa2 and nuclear receptors

Foxa2 has a role as a pioneer factor for nuclear hormone receptors in various organs and settings [98]. During fasting, Foxa2 is required for the target occupancy sites of the glucocorticoid receptor to promote hepatic gluconeogenesis [90]. More specifically, Foxa2 is required for stimulatory activity of glucocorticoid and cyclic AMP (cAMP) to promote the binding of cAMP response element-binding protein (CREB) and glucocorticoid receptor (GR) at their target sites [90]. In aged mice, Foxa2 occupancy has shown to be increased in aged and fatty livers, specifically in the binding regions of PPAR and liver X receptor (LXR), and cooperates with these nuclear receptors in the regulation of gene expression changes [99,100]. Foxa2 has also been reported to interact with androgen receptors in the male reproductive accessory organs [101]. In the epididymis, but not prostate, Foxa2 co-localized with the androgen response elements and directly interacts with the androgen receptor to regulate epididymis-specific genes [101].

1.8.3 Non-metabolic post-natal roles of FOXA2

In mammals, Foxa2 has a key role during embryogenesis and in the differentiation of the pancreas and liver in adult mice [83,85,86,90,92]. Besides metabolic regulation, Foxa2 has been linked to bile acid-induced inflammation and premature aging in the liver [102]. Mice with conditional Foxa2 knockout in liver exhibited an increased in bile acids accumulation which transduced pro-inflammatory cytokine signaling [102]. The premature aging phenotype in Foxa2-deficient mice was linked to the increased signaling of mammalian target of rapamycin (mTOR) pathway, through the reduced expression of mTOR repressors [102]. In nematodes, the transcription factor PHA-4/FoxA has also been previously reported to promote longevity through the similar inhibition of TOR signaling and caloric restriction [103].

The post-natal regulation of Foxa2 has also been reported in the epithelial cells of lungs, and in the enteroendocrine system [104,105]. Dysregulation of Foxa2 expression in these organs has been reported as a pathological prognostic factor in respiratory diseases and in various cancers [106,107]. In the lungs, Foxa2 is identified as a key regulator in normal post-natal pulmonary homeostasis and contributes to the regulation of innate immunity to aeroallergen exposure [105]. Foxa2 regulates and maintains healthy levels of airway mucus homeostasis, which contributes towards innate host defense against pathogens, particles, and toxicants [108]. Deletion of Foxa2 in mouse respiratory epithelial cells induced type 2 helper T cells (Th2) -mediated pulmonary inflammation through increased production of Th2 cytokines [105]. In the mammalian gastrointestinal tract, the lack of Foxa2 caused improper differentiation of the intestinal endocrine cells and the reduction of secretory mucins [104]. The decreased

in intestinal goblet and enteroendocrine cells and their functions have been implicated in intestinal inflammation and carcinogenesis [109,110].

1.9 Transcriptional regulation by peroxisome proliferator-activated receptors (PPARs)

Besides environmental variables and hormonal regulation, metabolic homeostasis in tissues and organs can be regulated through the control of metabolic gene expressions. In skeletal muscle, PPARs are important regulators of lipid and glucose metabolism [111]. PPARs are controlled by nutrient availability and, in turn, they control nutrient handling and the cellular signaling events [111]. PPARs belong to the ligand-activating nuclear hormone receptor superfamily, and they are activated by a variety of synthetic ligands and endogenous ligands, such as naturally occurring free fatty acids (FFA) and their derivatives, arachidonic acid and eicosanoids [112]. Synthetic pharmaceutical ligands of PPARs have also been used in the treatment of metabolic syndrome and T2DM, and thus are considered valuable pharmaceutical targets [113].

Three related PPAR family members, designated as PPAR α , PPAR β/δ , and PPAR γ , have been each identified [114]. The members of the PPAR family shared similar protein homologies but exhibit distinct functional roles in energy metabolism [115]. Moreover, each member of the PPAR family displays specific tissue distribution patterns and pharmacological profiles. PPAR α is found in high amounts in metabolic tissues, such as the liver, heart, and skeletal muscle [116]. While PPAR γ is mainly expressed in the adipose tissue, colon, and macrophage [117]. Two distinct PPAR γ

isoforms are detected in both human and mouse, namely PPAR γ 1 and PPAR γ 2 [118]. PPAR γ 1 is present in most tissue, whereas PPAR γ 2 is regulated by nutrition and its expression is restricted to the white and brown adipose tissue [119]. PPAR β/δ expression is found ubiquitously and is known to exert its importance in the systemic and basic cellular functions, which include the energy modulation in metabolically active tissues, inflammation, wound healing, and keratinocyte and intestinal cell differentiation [7,9]. The *PPARD* gene ablation in mice results in a high embryonic lethality [21], and the PPAR γ -deficient mice exhibit embryonic lethality by E10 [22]. All three of the PPARs are expressed in the skeletal muscle at different amounts, as follows: PPAR β/δ has the highest expression levels, followed by PPAR α and PPAR γ [23–25].

1.9.1 Structure of PPARs

The members of the PPAR family share modular structure characteristics with four distinct domains, similar as to most nuclear receptors [120]. The N-terminal contained the A/B domains which encodes the activation function 1 (AF1) that function independently of ligand activation; the C-domain consist two zinc finger-like structures encoded in their highly conserved DNA binding domain (DBD); the hinge region that provide structural flexibility; and the ligand binding domain (LBD) in the E-domain which contains the ligand-dependent activation function 2 (AF2) [121].

The LBD structure of PPARs forms helices bundle and stranded beta-sheet that delineates a large Y-shape hydrophobic pocket (about 1400 cubic angstroms) as compared to other nuclear receptors, allowing a wide range of natural and synthetic ligand interactions [122]. In comparison among the PPAR isotypes, the LBD of PPAR α

and PPAR γ are more similar in shape and size while PPAR β/δ LBD is significantly smaller [121,123]. The differences in amino acid sequences among the PPAR isotypes also revealed PPAR α LBD pocket is more lipophilic than the two others [123]. These structural differences among the PPARs LBD suggest the influences of structurally-distinct ligands with varying binding affinities, thereby contributing to ligand isotype selectivity [124].

All three PPARs members form obligate heterodimers with retinoid X receptors (RXRs), which bind as complex on specific consensus sequences located in the regulatory region of its target genes, known as the peroxisome proliferator response elements (PPRE) [125]. The PPRE is composed of a direct repeat (DR) AGGTCA-like sequences, directionally aligned with one or two nucleotide spacers (DR1 or DR2) [125]. PPARs can be activated by a variety of synthetic ligands and endogenous ligands, such as naturally occurring FFA and their metabolites, arachidonic acid and eicosanoids [126]. Upon ligand activation, conformational changes in PPARs induce the release of transcriptional repressors, and allow the binding of other activator proteins, such as CBP/p300 and histone acetyltransferases, leading to the upregulation of PPAR target gene transcription [127].

PPAR α and PPAR γ are not sufficiently stable in the absence of ligand [128]. In contrast, unliganded PPAR β/δ -RXR complexes can exert basal transcriptional repression, including PPAR α - and PPAR γ -dependent transactivation through competitive bindings of PPRE sites via strong interactions with co-repressor molecules [129]. In addition, the activity of PPARs may also be affected in the presence or absence of ligand by post-translational modifications, such as phosphorylation, SUMOylation

and ubiquitination [128]. Post-translational modification by ubiquitination has shown to be affected by the presence of PPAR ligand [128]. In the absence of ligand, PPAR α and PPAR β/δ are poly-ubiquitinated and targeted for subsequent degradation [130,131]. The presence of PPAR γ agonists, on the other hand, enhances PPAR γ polyubiquitination which promotes its degradation [132]. Ligand binding of PPAR β/δ is required for the inhibition of ubiquitination and the subsequent block of its degradation, thus suggesting an essential step to extend PPAR β/δ short half-life and sustain its transcription activity [128].

Figure 1.5 Transcriptional activation of target genes by PPAR.

domain which contains the AF2 ligand dependent activation domain. The D domain consists of a highly flexible hinge region.

1.10 Metabolic regulation by PPARs in skeletal muscle

PPARs are diverse regulators that fundamentally regulate the energy metabolism at the transcription level. The peroxisome proliferator-activated receptors (PPARs) are well known and have emerged as the master regulators of both lipid and glucose homeostasis [111]. The members of the PPAR family modulate metabolic responses through sensing and responding to fluctuations in the nutrient availability [133]. Major dietary constituents, such as fatty acids and carbohydrates, can regulate the gene expression of several metabolic pathways via hormones and PPARs and, in turn, induce their utilization [126]. In a post-prandial state, the availability of metabolic precursors promotes the synthesis of natural PPAR ligands and induces PPAR trans-regulation so as to promote anabolism and storage [134]. During fasting or nutrient scarcity, PPARs are directly activated by the released FFAs from lipid reserves, which stimulate the transcription of genes that are involved in fatty acid oxidation in the skeletal muscle, as well as glycogenolysis, gluconeogenesis, and ketone body synthesis in the liver [135]. During fasting, both PPAR α and PPAR β/δ are upregulated in skeletal muscle in rodents [136], but only PPAR β/δ is upregulated in human skeletal muscle [137,138]. FFA influx is increased, and FFAs are hydrolyzed into acyl-CoA complexes, which are then channeled into the mitochondria for fatty acid oxidation [137]. One of the key genes that regulate the glucose-FFA shuttle is pyruvate dehydrogenase kinase (PDK), which is a classical PPAR target gene [137]. PDK inactivates PDC via phosphorylation and reduces the oxidation of glycolysis-derived pyruvate [139]. These effects decrease glucose utilization to conserve glucose [137]. In human skeletal muscle, all four PDK

isozymes are PPAR β/δ target genes, and PDK2 and PDK4 are the most abundantly expressed [138,140]. In the skeletal muscle of PPAR β/δ knockout mice, the PDK4 expression is markedly blunted [141]. PDK4 expression is however unaffected in the skeletal muscle of fasted PPAR α knockout mice [142]. Thus suggesting PPAR β/δ is the primary PPAR member that regulates skeletal muscle substrate utilization.

In humans, skeletal muscle accounts for more than 30% of the total energy expenditure, and up to 70% of this energy is derived from FFAs in resting muscle [137]. Of the 3 PPAR members, PPAR α and PPAR β/δ play central roles in modulating cellular energy consumption and production [142]. PPAR γ promotes glucose uptake in skeletal muscle to play a role in insulin-stimulated glucose metabolism [143]. Overexpression and knockdown studies of PPAR α and PPAR β/δ in mice have exhibited their regulation on genes involved in FFA uptake, fatty acid oxidation and utilization [144-146]. In skeletal muscle, activation of PPAR β/δ increased FFA uptake and lipid catabolism via beta-oxidation [147]. Skeletal muscle-specific PPAR β/δ overexpression also induces characteristic shifts towards oxidative fibers, enhanced increased oxidative capacity, leading to an overall reduction in body fat [148]. Similarly, transcriptome analyses of rodent PPAR β/δ in skeletal muscle showed its importance in fatty acid transport, fatty acid beta-oxidation, and mitochondrial respiration [137]. Conversely, selective PPAR β/δ ablation in skeletal muscle leads to lower oxidative capacity in the fibers, resulting in obesity and T2DM [149]. PPAR α overexpression in mice, in contrast to PPAR β/δ , promotes fiber type shifts towards glycolytic type II fibers, which protected PPAR α -overexpressed mice from diet-induced obesity [137]. However, PPAR α -overexpressed mice developed glucose intolerance and have significantly higher intramuscular triglyceride concentrations than control mice when fed with a high-fat

diet [145]. PPAR α knockout mice, on the other hand, exhibited reduced fatty acid oxidation during starvation despite an increase in oxidative fibers [142].

1.11 Role of PPAR in insulin resistance and T2DM

In skeletal muscle, the major causes of insulin resistance are thought to be the excess accumulation of intramyocellular lipid and the inhibition of one or several steps in the insulin signaling cascade [150]. PPAR agonists have been of clinical interest since the discovery of fibrates and TZDs for treating metabolic-related diseases [151].

1.11.1 PPAR γ in insulin resistance and T2DM treatment

PPAR γ ligands, including TZDs, have hypoglycemic effects, reduce insulin resistance and improve insulin sensitivity [152]. TZDs were reported as insulin sensitizers in the 1980s [153]. Currently, pioglitazone is the only FDA-approved TZD for treating T2DM [152]. Pioglitazone is known to have lipid-modifying benefits and can reduce adverse cardiovascular outcomes [152]. The insulin-sensitizing effects of TZDs can be attributed to the activation of skeletal muscle PPAR γ [154]. This activation maintains insulin signaling activity even though PPAR γ is expressed at low levels [155]. Given the whole-body skeletal muscle mass, the regulation of skeletal muscle PPAR γ remains physiologically relevant [137]. The direct action of TZDs on non-adipose tissues has been indicated in adipose tissue-specific PPAR γ -silenced mice in which TZD treatment improved insulin sensitivity in skeletal muscle and liver despite an increase in triglyceride deposition [156]. In obese Zucker rats, short-term treatment with rosiglitazone, another well-known TZD, increases phosphorylation of the insulin receptor and IRS-1 as well as induces AKT activation in the skeletal muscle [157].

Similarly, muscle biopsies obtained from T2DM patients treated with either rosiglitazone or pioglitazone showed increased insulin-stimulated IRS-1 phosphorylation, PI 3-kinase (PI3K) phosphorylation cascade, and AKT activity [158,159]. In L6 muscle cells, PPAR γ agonists, but not PPAR α agonist, have shown to increase IRS1 protein expression directly [160]. Moreover, constitutive PPAR γ activation in mouse skeletal muscle decreases intramuscular lipid accumulation, induces a shift towards the oxidative fiber type and protects against susceptibility to diet-induced insulin resistance [161]. Conversely, skeletal muscle-specific PPAR γ knockout mice have increased adiposity and are glucose intolerant and insulin resistant [154,162]. In human, skeletal muscle PPAR γ is acutely regulated and increased by insulin [163]. PPAR γ activation directly regulates the expression of glucose transporters GLUT1 and GLUT4 and promotes their translocation to the cell surface to increase cellular glucose uptake [164]. On a side note, GLUT4 regulation by PPAR γ has been shown to be remarkably conserved across vertebrate evolution, from fish to mammals [165]. Dominant negative PPAR γ mutations in humans have been associated with obesity [166], dyslipidemia, and severe insulin resistance [167], whereas a common polymorphism (Pro12Ala) has been shown with reduced PPAR γ receptor activity, improved insulin sensitivity and decreased in T2DM risk [168,169].

1.11.2 PPAR α in insulin resistance and T2DM treatment

PPAR α plays a pivotal role in the liver during nutritional transitions and intricately controls hepatic lipid metabolism and whole-body glucose homeostasis [135]. The role of skeletal muscle PPAR α in regulating the insulin signaling pathway is, however, less clear [137]. Though PPAR α has metabolic regulatory roles, its expression in skeletal muscle remains unchanged during fasting [138]. The clinical use of fibrates

for treating hyperlipidemia in obese individuals and T2DM patients was first approved in the late 1960s [170]. The fibrates commonly used for clinical treatment are bezafibrate, fenofibrate, and gemfibrozil [171]. Fenofibrate treatment in patients with metabolic syndrome improves lipid profiles and increases insulin sensitivity [172,173]. Also, bezafibrate has been reported to increase skeletal muscle AKT phosphorylation and improve insulin sensitivity in insulin-deficient streptozotocin-treated mice [174]. However, both bezafibrate and fenofibrate exhibit weak PPAR β/δ and/or PPAR γ agonist activity [170,175]. The direct pharmacological activity of PPAR α on skeletal muscle insulin sensitivity thus remained elusive [137].

1.11.3 Potential role of PPAR β/δ in insulin resistance and T2DM treatment

PPAR β/δ agonists may be insulin sensitizers and have been suggested as a potential therapeutic approach for treating metabolic dysfunction and T2DM [176]. Currently, there are no PPAR β/δ agonists approved for clinical treatment, but several are in the development and clinical study phases. [113]. One prominent PPAR β/δ -selective agonist is seladelpar (MBX-8025), currently, in clinical phase 2/3 for primary biliary cirrhosis, has previously shown to improve insulin sensitivity and dyslipidemia in overweight subjects [177]. The well-known GW501516, though its development was halted in 2007, has since served as an important PPAR β/δ -specific agonist in the elucidation for PPAR β/δ physiological and pathophysiological functions [137]. In animal models of obesity and T2DM, PPAR β/δ activation through specific agonists or genetic manipulation ameliorates hyperglycemia, insulin resistance, and dyslipidemia [137]. PPAR β/δ silencing renders mice glucose intolerant and less metabolically active [178]. Similarly, skeletal muscle-specific PPAR β/δ knockout mice exhibit insulin insensitivity and impaired glucose tolerance [149]. PPAR β/δ agonist treatment

improves whole-body insulin sensitivity through complementary actions in the skeletal muscle and liver [176]. In insulin-resistant obese mice, activating PPAR β/δ through GW501516 ameliorates hyperglycemia-mediated glycolysis and lipogenesis increases in the liver to reduce hepatic glucose output [176]. Simultaneously, GW501516 is known to promote fatty acid oxidation, to enhance skeletal muscle insulin sensitivity, and reduce circulating triglyceride levels [146,178].

1.12 Non-metabolic role of PPARs

Besides cellular metabolism, PPARs are also known to be involved numerous systemic and cellular activities [179]. Members of the PPAR family are known to be involved in inflammation, tissue repair as well as tumor progression. PPARs are central effectors to pro-inflammatory induction and/or modulation, which have been known to serve protection against external infections and pathological-related inflammation induced by type 2 diabetes and atherosclerosis [179]. Mechanistically, PPARs are known to exert their anti-inflammatory effects through direct interactions with inflammatory transcription factors such as NF- κ B and STAT, resulting in the co-repressors recruitment or the inhibition of coactivators recruitment [180]. PPAR α expression is transiently upregulated during injury in mice and have been reported of its role in suppressing inflammation [181]. In the liver and smooth muscle cells, PPAR α agonist have also been shown to inhibits nuclear translocation of NF- κ B and thus reduces NF- κ B pro-inflammatory actions [182]. Comparatively, activation of PPAR γ leads to a reduction in cytokine secretion, including TNF α and IL6 [183]. PPAR γ have been shown to interfere with the c-Jun and NF- κ B localization in specific cell types such as colonic epithelial cells, macrophages and vascular endothelial cells [184-186].

In contrast to PPAR α and PPAR γ , the modulation of inflammation by PPAR β/δ is the least understood and remained to be fully elucidated [179]. Transrepression mediated by PPAR β/δ and the recruitment of the transcriptional repressor BCL-6 during the absence of PPAR β/δ ligand have been reported [179]. In the presence of ligand, PPAR β/δ exerts anti-inflammatory activity through the release of BCL-6, and represses NF- κ B-mediated gene transcription [187]. Anti-diabetic functions of PPAR β/δ have been associated with reduced inflammatory signaling and pro-inflammatory cytokine production, in conjunction with reduced hepatic fat accumulation [188]. In addition, PPAR β/δ have been shown as a key mediator in the reduction of exercise-induced vascular inflammation, of which it was not exhibited in PPAR β/δ -knockout mice [189].

1.13 Aim and objectives of the study

Postnatal expression of Foxa2 have been established as an important metabolic regulatory gene in the liver, pancreas and adipose tissue. Foxa2 ablation and overexpression studies have suggested its regulation of genes involved lipid oxidation and glycolysis, and that hormonal regulation of Foxa2 by glucocorticoids and insulin have been described. Little to none is however known about the role of Foxa2 in skeletal muscle. As of current writing of this thesis, there are no published reports on Foxa2 expression and its functional role in skeletal muscle. It is also not known if Foxa2 is involved in the downstream signaling of hormones in skeletal muscle. **We thus hypothesized that Foxa2 expresses in skeletal muscle and likely to be involved in lipid metabolism.** Moreover, Foxa2 has been positively associated with PPAR α and PPAR γ , as well as in their transcriptional regulation of metabolism. PPAR β/δ , the master regulator of energy metabolism in skeletal muscle, has yet been reported to interact with Foxa2.

Here we aim to first elucidate the expression of Foxa2 in skeletal muscle both *in vitro* and *in vivo*, and delineate the role of Foxa2 in skeletal muscle which include the effects of insulin and glucocorticoids in C2C12 myotubes. We will also identify potential pathogenic mechanism of Foxa2 dysregulation in skeletal muscle through gene manipulation techniques and investigate the potential PPAR β/δ regulation on Foxa2 expression.

CHAPTER 2. MATERIALS AND METHODS

The experimental procedures and reagents used in this study are described in the following sections.

2.1 Animal studies

Wild-type C57BL/6J mice were purchased from In Vivos, Singapore, and were housed in the specific pathogen-free facility with 12 h/ 12 h light/dark cycle with ad libitum access to chow diet and water. All experiments were performed on animals of 3 to 5 months of age, in accordance with the guidelines of the University's Institutional Animal Care and Use Committee, Singapore (IACUC). Mice were euthanized through CO₂ inhalation. M. quadriceps (Quad), M. gastrocnemius (Gas), M. tibialis anterior (TA) and M. soleus (Sol) of the hind limb skeletal muscle were dissected and snapped frozen in liquid nitrogen for subsequent biochemical analysis or embedded in Tissue-Tek® optimum cutting compound (OCT; VWR, USA) using liquid nitrogen cooled isopentane (Sigma Aldrich, USA) for subsequent histological analysis.

Injury of hindlimb Quad and TA muscle was performed through the injection of 1% λ -carrageenan solution (2 mg/ 100 μ L saline) at 10 mg/kg body weight. The same volume of sterile saline solution was injected into the contralateral TA muscle which served as a control. Treatment of hindlimb muscle with PPAR β/δ agonist GW501516 was performed immediately upon injury at 15 mg/ mL/ kg body weight.

Systemic inflammation was induced in wild type mice through the intraperitoneal injection with lipopolysaccharide (LPS; Sigma-Aldrich). LPS was diluted in saline and injected at 0.1 μ g per gram of mice weight. Mice were sacrificed 2 h after the injection.

The high-fat diet study was performed in 6-weeks old male mice, along with litter-batch mice fed with control diet for 8 weeks. One week prior to harvesting, oral glucose tolerance test was performed. All animal maintenance, feeding and animal testing were performed by another PhD student, Mr Low Zun Siong. On the day of harvest, quadricep muscles were collected and snapped frozen in liquid nitrogen and stored in -80°C for subsequent analysis.

2.2 Cell culture

C2C12 mouse myoblasts were obtained from the American type culture collection (ATCC; USA) and grown in less than 70% confluency in proliferation medium (Table 2.2.1). For myoblast studies, C2C12 myoblasts were seeded at the density of 15,000 cells/cm² in proliferation medium. To induce differentiation of myoblasts into myotubes, C2C12 myoblasts were first seeded overnight at the density of 25,000 cells/cm² in proliferation medium. Myogenic differentiation was induced by switching to differentiation medium (Table 2.2.2) on the next day. The first day of incubation in differentiation medium was defined as “day 0 of differentiation”. All myotubes studies were performed after 3 days of differentiation, as previously validated and reported in [190]. Cells were at 37°C under humidified incubator with 5% (v/v) CO₂. All cell culture treatments were performed in fresh serum-free alone medium (Table 2.2.3) and changed every day, unless specified.

Table 2.2.1 Proliferation medium

Chemicals	Concentration
DMEM (with 1 mM sodium pyruvate)	
Glucose	4.5 g/L
Sodium bicarbonate	3.7 g/L
Fetal bovine serum	10% (v/v)
pH 7.4	

Table 2.2.2 C2C12 differentiation medium

Chemicals	Concentration
DMEM (with 1 mM sodium pyruvate)	
Glucose	4.5 g/L
Sodium bicarbonate	3.7 g/L
Horse serum	2%
pH 7.4	

Table 2.2.3 DMEM serum-free medium

Chemicals	Concentration
DMEM (with 1 mM sodium pyruvate)	
Glucose	1.0 g/L
Sodium bicarbonate	3.7 g/L
pH 7.4	

2.3 Insulin treatment in C2C12 myotubes

A stock solution of 10 mM was prepared insulin from bovine pancreas (Signal-Aldrich, USA) in PBS and stored in -20 °C. Cells were washed twice with PBS and incubated with serum-free MEM α medium (Hyclone, USA), prior to insulin stimulation. Insulin treatment was performed on the 3rd day of C2C12 differentiation at various concentrations in serum-free MEM α medium (Sigma-Aldrich, USA) for 24 h.

2.4 Glucocorticoid treatment in C2C12 myotubes

A stock concentration of 10 mM dexamethasone (Dex; Signal-Aldrich, USA) and hydrocortisone (Cort; Signal-Aldrich, USA) were prepared in DMSO and stored in -20 °C prior to treatment. Dex and Cort treatment was performed on the 3rd day of C2C12 differentiation at a concentration of 1 nM in serum-free DMEM medium for 24 h. Control cells were incubated with 0.001% (v/v) DMSO.

2.5 High fatty acid treatment in C2C12 myotubes

A stock solution of 50 mM sodium palmitate (PA; Sigma Aldrich, USA) was prepared in 50% ethanol, mixed until dissolved and stored at -20°C, while 50 mM oleate acid (OA; Sigma Aldrich, USA) was freshly prepared and dissolved in 100% ethanol. Fatty acid-free bovine serum albumin (BSA) was dissolved in serum-free DMEM media to a total concentration of 2% (w/v). Fatty acid conjugation in 2% BSA media was prepared by diluting PA or OA in 2% BSA media and incubated for 15 minutes at 37°C. Fatty acid-BSA conjugate solutions were sterile filtered and added in dropwise to

differentiated myotubes. Control cells were incubated with less than 0.01% (v/v) ethanol.

2.6 LPS treatment in C2C12

A stock solution of 100 ng/uL of LPS was prepared in PBS and stored at 4°C. Cells were washed twice with PBS and incubated in 100 ng/mL LPS in serum-free DMEM media for 24 h.

2.7 Transient transfection of plasmid into C2C12 myoblasts

Myoblasts were seeded overnight prior to transfection at a density of 15,000 cells/cm² in proliferation medium. Cells were washed twice with phosphate-buffered saline before transfection. The transfection medium was prepared in 4:1 FuGene HD transfection reagent (Promega, USA) to DNA ratio, according to the manufacturer's protocol. Briefly, 1:1 DNA ratio of shFoxa2-A and shFoxa2-B plasmid DNA was diluted in C2C12 serum-free medium before the addition of FuGene HD transfection reagent (Promega, USA). Upon 10 minutes of incubation at room temperature, the transfection mixture was top up with proliferation medium required per well, before adding into the cells dropwise. Transfected cells were incubated for 24 to 48 h prior to analysis.

Table 2.7.1 Foxa2 shRNA target sequence

Mouse shFoxa2	Target nucleotide sequence (5' to 3')
shFoxa2-A	GCACAAGCGAGGTGGCCTA
shFoxa2-B	TGAATGGCATGAACACATA

2.8 RNA isolation and cDNA synthesis

Total RNA was extracted from cells and tissues using TRIzol (Invitrogen, USA) and purified with e.Z.N.A Total RNA Kit (Omega Bio-Tek, USA). Samples were incubated for 5 minutes at room temperature to allow complete dissociation of the nucleoprotein complex. For every 1 mL TRIzol used, 0.2 mL chloroform was added. Tubes were shaken vigorously by hand for 15 seconds and further incubated for 3 minutes at room temperature. Samples were then centrifuged at 13,000g for 15 minutes at 4°C. The aqueous phase was transferred to a new tube, mixed with 0.5 mL isopropanol per mL TRIzol used and incubated for another 10 minutes. For RNA purification, samples were then transferred into mini-columns provided in the kit, washed thrice as per manufacturer's recommendations and eluted in 50 µL DEPC water. The RNA purity and quantity were measured using Nanodrop ND1000 (Thermo Scientific, USA). Reverse transcription of RNA was performed with qScript cDNA SuperMix (Quanta Biosciences, USA) according to manufacturer's protocol in iCycler thermal PCR System (Bio-Rad Laboratories, USA). The cDNA was diluted with deionized water and stored in -20°C until used.

2.9 Quantitative Real-time PCR analysis (qRT-PCR)

Quantitative real-time PCR (qPCR) reactions were prepared in triplicates with PerfeCTa SYBR Green FastMix (Quanta Bio, USA) according to manufacturer's protocol, and analyzed using C1000 Thermal Cycler with CFX96 Real-Time System module (Bio-Rad Laboratories, USA). Oligonucleotides were designed using NCBI primer designing, purchased from Sigma Aldrich, Singapore, and tabulated in Table 2.9.1.

Table 2.9.1 Primer sequences of mouse genes probed in qPCR assays

Gene (mouse)	Forward (5' to 3')	Reverse (5' to 3')
Lpl	ATGGATGGACGGTAACGGGAA	CCCGATACAACCACTCTACA
Dlk1	AGTGCGAAACCTGGGTGTC	GCCTCCTTGTTGAAAGTGGTCA
Fabp5	TGAAAGAGCTAGGAGTAGGACTG	CTCTCGGTTTTGACCGTGATG
Hk2	TGATCGCCTGCTTATTCACGG	AACCGCCTAGAAATCTCCAGA
Igfbp5	AAGACAGCACAACTTCAGTTCA	TCACAGGGAAGGATGTTTGAGT
Angptl4	CCCCACGCACCTAGACAATG	GCCTCCATCTGAAGTCATCTCA
Tnfa	CCCTCACACTCAGATCATCTTC	GCTACGACGTGGGCTACAG
Il6	TCTATACCACTTCACAAGTCGGA	GAATTGCCATTGCACAACTCTTT
Cxcl10	CCAAGTGCTGCCGTCATTTTC	GGCTCGCAGGGATGATTTCAA
Mcp1	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT
Cpt1a	CAGACTCGGTCACCACTCAAG	GAGATCGATGCCATC AGGGG
Foxa2	GAGCACCATTACGCCTTCAAC	AGGCCTTGAGGTCCATTTTGT
Cd36	ATTGGTCAAGCCAGCT	TGTAGGCTCATCCACTAC
Fabp3	CATGTGCAGAAGTGGAACGG	CTCACCACACTGCCATGAGT
Ppara	ACTACGGAGTTCACGCATGTG	TTGTCGTACACCAGCTTCAGC
Ppard	CGGCAGCCTCAACATGG	AGATCCGATCGCACTTCTCATAC
Glut4 (Slc2a4)	TCATTGTCGGCATGGGTTT	GGCAAATAGAAGGAAGACGTAAGG
Pdk4	GAGGATTACTGACCGCTCTTTAG	TTCCGGGAATTGTCCATCAC
Mmp9	TGTACCGCTATGGTTACACTCG	GGCAGGGACAGTTGCTTCT
Atrogin	TGTGGGTGTATCGGATGGAGA	CTGCATGATGTTTCAAGTTGTAAG
MuRF1	ACGAGAAGAAGAGCGAGCTG	CTTGGCACTTGAGAGGAAGG
Mstn	TGACGATTATCACGCTACCACG	GTAGGAGTCTTGACGGGTCTG

2.10 Protein extraction and immunoblot analysis

Samples were extracted in cold protein lysis buffer and centrifuged at 12,000 g for 10 minutes. The supernatant containing the protein extract was retrieved for immunoblot. Equal amount of protein extracts were resolved in SDS polyacrylamide gel electrophoresis (SDS-PAGE) using Mini-PROTEAN II electrophoresis cell (Bio-rad Laboratories, USA). Protein was then electrotransferred to a low-fluorescent PVDF membrane (Merck Millipore, USA) under constant voltage for 1 h with chilled transfer buffer. The membrane was blocked for 1 h at room temperature with Odyssey Blocking Buffer (LI-COR Biotechnology, USA). The membrane was then probed with the respective primary antibody overnight at 4°C in blocking buffer. The membrane was washed for 5 minutes thrice with TBS and incubated with appropriate secondary antibody in TBS containing 0.5X blocking buffer 1 h at room temperature. Excess secondary antibody was then washed off, and the membrane was dried before being analyzed using the Odyssey CLx scanner and Image Studio V2.1 (LI-COR Biosciences, USA).

Table 2.10.1 Protein lysis buffer

Chemicals	Concentration
Na ₂ HPO ₄	20 mM
NaCl	250 mM
Triton-X	0.1% v/v
SDS	0.1% w/v
PMSF	1 mM
Na ₃ VO ₄	100 µM

Table 2.10.2 Protein loading dye (6X)

Chemicals	Concentration
Tris-HCl (pH 6.8)	0.35 M
10% SDS	250 mM
Bromophenol blue	0.4%
β-mercaptoethanol	5% v/v
Glycerol	Top up to required volume

Table 2.10.3 4% Bis-acrylamide stacking gel

Chemicals	Volume
30% Acrylamide: Bis (29:1)	0.67 mL
1.0 M Tris-HCl, pH 6.8	0.5 mL
10% SDS	0.04 mL
10% ammonium persulfate	0.04 mL
TEMED	4 μL
Milli-Q H₂O	2.7 mL

Table 2.10.4 10% Bis-acrylamide running gel

Chemicals	Volume
30% Acrylamide: Bis (29:1)	3.3 mL
1.5 M Tris-HCl, pH 8.8	3.3 mL
10% SDS	0.1 mL
10% ammonium persulfate	0.1 mL
TEMED	8 μL
Milli-Q H₂O	4 mL

Table 2.10.5 Tris-glycine-SDS running buffer

Chemicals	Concentration
Tris	25 mM
Glycine	192 mM
SDS	0.1% w/v
Milli-Q H ₂ O	Top up to 1 L

Table 2.10.6 Western blot transfer buffer

Chemicals	Concentration
Tris	25 mM
Glycine	190 mM
Methanol	20% v/v
Milli-Q H ₂ O	Top up to 1 L

Table 2.10.7 Tris-buffered saline

Chemicals	
Tris-HCl, pH 7.5	50 mM
NaCl	150 mM
pH 7.5	

Table 2.10.8 Antibodies used

Chemicals	Company	Catalog number	2° Ab used
Foxa2	Cell Signalling Technology	D56D6	Rabbit
MyH	DSHB	D20	Mouse
MyL	DSHB	T14	Mouse
p-Akt (Ser 473)	Santa-Cruz, USA	SC-7985R	Rabbit
Akt (H-136)	Santa-Cruz, USA	SC-8312	Rabbit
MyoD	Santa-Cruz, USA	C-20	Rabbit
MyoG	Santa-Cruz, USA	M-225	Rabbit
PPARβ/δ	Santa-Cruz, USA	SC-7197	Rabbit
β-tubulin	Developmental Studies Hybridoma Bank, USA	E7	Mouse
Actin	Santa-Cruz	Sc-32251	Mouse
Myc Tag	Sino Biological	100029-MM08	Mouse
IRDye® 680LT goat anti-mouse	LI-COR Biosciences, USA		
IRDye® 680LT goat anti-rabbit	LI-COR Biosciences, USA		

2.11 Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed with minor modifications from a previously described protocol (Chong et al., 2009). Briefly, 1×10^6 cells were seeded overnight prior to $1 \mu\text{M}$ GW501516 treatment for 6 hours. Cells were crosslinked using 0.5% formaldehyde for 10 minutes at 37°C and harvested for sonication in lysis buffer (Table 2.11.1) to obtain cross-linked DNA measuring 200-500 bp in length. Sonicated DNA lysate was diluted 10-fold in ChIP dilution buffer (Table 2.11.2). 1% of the diluted supernatant was retained for input loading while the remaining portion was divided proportionately and subjected to ChIP with monoclonal anti-PPAR β/δ antibody (Santa-Cruz Biotechnology, USA), monoclonal anti-FoxA2 antibody (Cell Signaling Technology, USA) or monoclonal anti-IgG antibody (Santa-Cruz Biotechnology, USA). Complexes that formed upon overnight incubation at 4°C were then pulled down using Protein A agarose/salmon sperm DNA (Millipore, USA). Beaded agarose resins were washed thrice, with the first wash in low salt immune complex wash buffer (Table 2.11.3), then in high salt immune complex wash buffer (Table 2.11.4), and finally Tris-EDTA buffer (2.11.5). The bound complexes were then eluted in the elution buffer. DNA fragments were subsequently reverse crosslinked at 65°C for 6 h.

The reagents used during ChIP assay are listed as follow:

Table 2.11.1 ChIP lysis buffer

Chemicals	Concentration
Tris-HCl, pH 8.1	50 mM
EDTA	10 mM
SDS	1% w/v
pH 8.1	

Table 2.11.2 ChIP dilution buffer

Chemicals	Concentration
Tris-HCl, pH 8.1	16.7 mM
NaCl	167 mM
EDTA	1.2 mM
Triton X-100	1.1% v/v
SDS	0.01% w/v
PMSF	1 mM
pH 8.1	

Table 2.11.3 Low salt immune complex wash buffer

Chemicals	Concentration
Tris-HCl, pH 8.1	20 mM
NaCl	150 mM
EDTA	2 mM
Triton X-100	1% v/v
pH 8.1	

Table 2.11.4 High salt immune complex wash buffer

Chemicals	Concentration
Tris-HCl, pH 8.1	20 mM
NaCl	500 mM
EDTA	2 mM
Triton X-100	1% v/v
pH 8.1	

Table 2.11.5 Tris-EDTA buffer

Chemicals	Concentration
Tris-HCl, pH 8.1	20 mM
EDTA	2 mM
pH 8.1	

Table 2.11.6 ChIP elution buffer

Chemicals	Concentration
NaHCO ₃	0.1 M

2.12 DNA purification and amplification

DNA fragments obtained from the reverse cross-linking step in ChIP assay were purified using E.Z.N.A Gel Extraction Kit (Omega Bio-Tek, USA) according to the manufacturer's protocol. The DNA fragments were mixed in XP2 binding buffer 1:1 volume ratio, then transferred to a DNA mini column. Samples were washed twice with the provided SPW wash buffer and eluted in 40 µL of elution buffer.

Purified DNA was then amplified through polymerase chain reaction (PCR). Each amplification reaction contained 10 µM PCRBIO Ultra Mix (PCR Biosystems, UK), 0.4 µM of forward and reverse primers, and DNA. The primer pairs used for the ChIP assay were shown in Table 2.12.1.

Table 2.12.1 ChIP primer sequence

ChIP Primers	Forward (5' to 3')	Reverse (5' to 3')
Foxa2 PPRE1	CTTTGGGCACCTTGGATTTA	AAGCAGGTGACAGCCAATTT
Foxa2 PPRE2	CCAGAGGACTTGGTGTGGT	TCATGTTGCTCACGGAAGAG
Negative PPRE Control	GGAGAGTGGGCAATGGAATA	TGCTTAACGCTGTGCAATTC
Il6 promoter	TTACCCACCTGGCAACTCCT	TGCTTGTTTTCTGTGTCTTG

2.13 DNA agarose gel electrophoresis

1% (w/v) agarose gel was prepared in 1x TAE buffer, boiled for 1 minute or until completely dissolved. The mixture was then cooled under running tap before 1 μ L FloroSafe DNA stain (1st BASE, Singapore) per 10 mL TAE buffer/agarose mixture was added. The DNA agarose gel was then cast in a horizontal gel electrophoresis system (Bio-Rad, USA). Samples were mixed with 6x DNA gel loading dye (Thermo Fisher Scientific, USA) to a final concentration of 1x before loading into the gel. The DNA gel was then resolved at 100V for 45 minutes.

The composition of the reagents used for DNA gel electrophoresis is listed as follow.

Table 2.13.1 TAE buffer

Chemicals	Concentration
Tris-HCl (pH 7.6)	40 mM
EDTA	1 mM
Acetic acid	20 mM

2.14 Transcription reporter gene assay

Transcriptional reporter assays were performed in HEK293T cells using luciferase reporter plasmids along with expression constructs and firefly luciferase pGL2-promoter vector (Promega, USA) as internal control. Reporter plasmids were transfected into the cells plated on 6-well plate using Fugene HD transfection reagent (Promega). Total plasmids were kept at 1 µg-per-well. After 16 hours of transfection, the cells were incubated with 1 µM and 10 µM GW501516 for 6 hours. DMSO was used as a vehicle control. Luciferase assays were then performed with a luciferase assays system (Promega). Cells were washed with PBS and harvested in Passive Lysis Buffer provided in the kit. Enzymatic activity was measured in each sample on GloMax 20/20 luminometer (Promega) according to manufacturer's protocol.

2.15 Transcription reporter gene assay plasmids

The transcription reporter assay constructs *PPRE1-luc* and Δ *PPRE-luc* were generated by insertion of artificial promoter sequence between the KpnI and XhoI sites of pGL2-promoter vector (Promega). Both artificial PPRE nucleotide sequences inserts (see Table 2.15.1) were synthesized, cloned into the vector and verified (BioBasic Inc, Singapore). The Δ PPRE1 insert construct contained two nucleotide base pair mutation which were generated through gene synthesis as well. The gene sequence alignment reports for both inserts are attached in appendix 5.

Table 2.15.1 Nucleotide sequences of inserts for luciferase reporter assay constructs

Insert	Gene product sequence (5' - 3')
PPRE1	cggggtacccacctactgccctgtttgttttagttacgaaatgctttgggcaccttgatttaactgaaaa gtaaccttgaaacaccgaggccctcatgccagaggcaaatacgctgcctcccggtattggctgcagctaa acgggtctctccaggccgactgaggtgggtagccagaaagaggactgaggttaactgacgaccagggcg gccagaccacgcgagtcctacgcgcctcctgaggccgccccgggacttaactgtaacggggaggggcct ccggagcagcggccagcgagttaaagggtgtgtacacagttttctaaataggacagcgctttgcaaattg gctgtcacctgcttgttgtttgacagggaatgtgcactaaaaggaggagaaacccgagataaataaatct ggcttgctcgtctcgcgagcgg
ΔPPRE1	cggggtacccacctactgccctgtttgttttagttacgaaatgctttgggcaccttgatttaactgaaaa gtaaccttgaaacaccgaggccctcatgccagaggcaaatacgctgcctcccggtattggctgcagctaa acgggtctctccaggccgactgaggtgggtagccagaaagagtcctgaggttaactgacgaccagggcg gccagaccacgcgagtcctacgcgcctcctgaggccgccccgggacttaactgtaacggggaggggcct ccggagcagcggccagcgagttaaagggtgtgtacacagttttctaaataggacagcgctttgcaaattg gctgtcacctgcttgttgtttgacagggaatgtgcactaaaaggaggagaaacccgagataaataaatct ggcttgctcgtctcgcgagcgg

2.16 Cytokine detection by bead-based multiplex immunoassay

Mouse skeletal muscle, m. quadriceps were dissected and lysed in mammalian protein extraction reagent (m-Per; Thermo Fisher Scientific Inc., USA) supplemented with complete protease inhibitor mix (Roche Applied Science, Germany). Samples were sent to a multiplex immunoassay service (Discovery Assay; Eve Technologies, Canada). The multiplex immunoassays were completed on a mouse cytokine kit (EMD Millipore,

USA) and readings were recorded on the BioPlex 200 system (Bio-Rad Laboratories, Inc., USA). The sensitivities of the assay ranged from 0.1 to 33.3 pg/mL.

2.17 Tissue sectioning and histological staining

Cross-sectional sections of TA muscle were cut at 14 μ m using a cryostat (Leica CM1950, Germany) and mounted on Superfrost Plus Microscope Slides (Thermo Fisher Scientific, USA). Muscle sections were washed in PBS for 15 minutes prior to staining. Muscle sections were stained as per described below (Table 2.17.1). The stained tissue sections were imaged using slide scanner microscope Zeiss Axio Scan Z1 (Carl Zeiss, Germany).

Table 2.17.1 Muscle sections H&E staining procedure

Reagent	Duration of exposure
Hematoxylin	45 seconds
Rinse with tap water	Until clear
1% HCl	3 dips
Rinse with tap water	Until clear
Eosin	3 minutes
95% Ethanol	5 minutes x 3
100% Ethanol	5 minutes x 3
Xylene	5 minutes x 3
Mount with DPX	Dry overnight

2.18 Immunofluorescence

C2C12 myotubes transfected with either vehicle, shFoxa2 vectors and/or Foxa2-myc tagged vector for 2 days in proliferation media and differentiated in differentiation media for 7 days prior to fixation. Cells were fixed with 1% paraformaldehyde (PFA; Sigma Aldrich, USA) for 15 minutes, washed and permeabilized in 0.1% Triton X-100 (Sigma Aldrich, USA) for 5 mins. Cells were then blocked in 3% FBS in PBS for 30 minutes at room temperature (RT). Incubation with the primary antibody was performed for 1 h at RT, cells were washed three times and incubated with the secondary antibody for 30 min at RT. The antibodies were used as the following: mouse anti-Myosin Heavy Chain (1:20, MF20, DSHB) and anti-mouse secondary antibody Alexa Fluor 594 conjugated (1:100, Life Technologies A-11001, USA). The cells were washed three times and nuclei were counterstained with Hoechst 33258 (1 mg/ml; Abcam, USA). Images were acquired with Zeiss Axio Observer Z1 (Carl Zeiss, Germany).

2.19 Statistical analysis

Statistical analyses were performed using either two-tailed student's T-test for group treatment in C2C12, two-tailed non-parametric Mann-Whitney test for 2 variables, or otherwise as stated in figure legends. All statistical tests were performed using GraphPad Prism software (version 6; GraphPad Software Inc, USA), with p values of < 0.05 considered significant. (*P < 0.05; **P < 0.01; N.S., not significant.)

CHAPTER 3: RESULTS

Characterization of Foxa2 in skeletal muscle

3.1 Foxa2 is expressed in skeletal muscle

We first examined the expression of Foxa2 in skeletal muscle to reveal a possible role for Foxa2 in skeletal muscle physiology. *In vitro*, Foxa2 was detected in C2C12, a primary line of murine myoblast that is often used as a myoblast model in the study of skeletal muscle biology, as it mimics *in vitro* and *in vivo* differentiation process [191] and is frequently used to study intracellular signaling in skeletal muscle cells [192]. The expression of Foxa2 in C2C12 remains unchanged over 72 h of differentiation, suggesting that Foxa2 was not involved in muscle differentiation (Figure 3.1A). Next, we examined the expression of Foxa2 in the various muscle type from the hindlimb of mice. Its expression was varied in different skeletal muscles, with a higher level in muscles with a larger proportion of type II fast-twitch fibers, such as gastrocnemius (Gas), quadriceps (Quad) and tibialis anterior (TA) compared with type I slow twitch fibers such as soleus (Sol) (Figure 3.1B). For further mechanistic analysis, we mainly used TA and Quad for analysis due to its fiber compositions and their anterior anatomical position. These observations suggest that Foxa2 may play a role in skeletal muscle physiology.

Figure 3.1 Foxa2 is expressed in skeletal muscle.

(A) Representative immunoblot of Foxa2, MyoD, myogenin, myosin heavy chain (MyH), myosin light chain (MyL) in C2C12 over 72 h of differentiation. β -tubulin was used as loading control. (B) Densitometric analysis of Foxa2 in C2C12 over 72 h of C2C12 differentiation. (C) Representative immunoblot of Foxa2 in various mice hindlimb muscles. β -tubulin was used as loading control. (D) Densitometric analysis of relative Foxa2 levels in C57B6J wild-type mice quadriceps; Quad, tibialis anterior; TA, gastrocnemius; Gas, and soleus; Sol. (Multiple comparisons one-way ANOVA test; $n=3$) The data are presented as the mean $\pm$ SEM from 3 independent experiments. * $P < 0.05$; ** $P < 0.01$; N.S., not significant.

3.2 Skeletal muscle Foxa2 expression is affected by saturated fatty acid but not glucocorticoids and insulin

Hormones are key regulators in the maintenance of the homeostatic balance in skeletal muscle metabolism during growth and health [193]. Insulin and glucocorticoids were previously shown to exhibit reciprocal effects on hepatic Foxa2 expression [90,91]. In skeletal muscle, the major hormonal effects of insulin promote carbohydrate metabolism via insulin-dependent Akt activation [194]. Glucocorticoids, on the other hand, are known to regulate protein metabolism and induce direct catabolic effects [195]. Elevated levels of glucocorticoids on skeletal muscle induce to muscle atrophy and loss of function [195,196]. To examine if Foxa2 expression in skeletal muscle was regulated through hormonal stimulation, C2C12 myotubes were exposed to either insulin, dexamethasone (Dex), hydrocortisone (Cort) for 24 h. Foxa2 transcriptional inhibition by insulin and insulin-dependent Akt activation has been previously reported in the liver and HepG2 cells [91,197]. In C2C12 myotubes, increasing concentration of insulin stimulation over 24 hours did not alter the mRNA and protein expression of muscle Foxa2 (figure 3.2 A, B). Similarly, Foxa2 mRNA and protein levels in myotubes did not alter over 24 hours stimulation with a low concentration of either cortisone or dexamethasone, without inducing excessive muscle atrophy-related genes (Atrogin and MuRF1) and the muscle negative regulatory factor myostatin (Figure 3.1 C, D).

To this end, we examined the effects of fatty acid loading on Foxa2 expression in C2C12 cells as fatty acids have emerged as signaling molecules that influence biological processes in recent years [198]. C2C12 myotubes were treated with increasing concentrations of either saturated or non-saturated fatty acids for 24 hours.

Saturated palmitic acid (PA) treatment increased Foxa2 expression at 500 μ M while unsaturated oleic acid (OA) did not alter Foxa2 mRNA expression at both 250 μ M and 500 μ M (Figure 3.2E). Palmitic acid, but not oleic acid, increased Il6 expression (Figure 3.2F), a myokine that has been associated with inflammatory signaling and energy regulation in skeletal muscle [199]. Western blot analysis of PA treatment at 500 μ M confirmed an increased Foxa2 protein expression by 1.4 folds (Figure 3.2F).

The expression of Foxa2 in skeletal muscle was neither affected by insulin nor glucocorticoids, which contrasted with its regulation in the liver. Foxa2 expression was increased by saturated fatty acid loading, but not unsaturated fatty acid in myotubes. Our results suggest a tissue-specific regulatory mechanism of Foxa2 in skeletal muscle.

Figure 3.2 Expression analyses of Foxa2 in C2C12 myotubes treated with insulin, glucocorticoids, and fatty acids.

(A) Gene expression analyses of Foxa2 and 6 other genes associated with muscle atrophic genes and energy metabolism in C2C12 myotubes treated with either 1 nM hydrocortisone (Cort) or 1 nM dexamethasone (Dex) for 24 h. DMSO served as vehicle control. (B) Representative immunoblot analyses of Foxa2, p-Akt and Akt in myotubes treated with either 1 nM Cort or Dex for 24 h. β -tubulin was used as loading control. (C) Fold change of Foxa2 mRNA expression analyses in C2C12 myotubes treated with increasing concentration of insulin for 24 h. (D) Immunoblot analyses of Foxa2, p-Akt and Akt in myotubes treated with various concentration of insulin for 24 hours. β -tubulin was used as loading control. (E) Foxa2 mRNA expression analyses in C2C12 myotubes treated with increasing concentration of oleic acid (OA) or palmitic acid (PA) for 24 h. Ethanol was used as vehicle control. (F) Il6 mRNA expression analyses in C2C12 myotubes treated with increasing concentration of oleic acid (OA) or palmitic acid (PA) for 24 h. Ethanol was used as vehicle control. (G) Representative immunoblot analyses of Foxa2, p-Akt, Akt in myotubes treated with 250 μ M or 500 μ M PA. Ethanol was used as vehicle control. (H) Densitometric analysis of Foxa2 in PA-treated myotubes. All data are presented as the mean $\pm$ SEM from 3 independent experiments. * $P < 0.05$; n.s., not significant.

3.3 PPAR β/δ directly regulates Foxa2 in skeletal muscle

Palmitate or its derivatives have been shown to be agonists of nuclear receptor PPAR [200]. As palmitate increased the expression of Foxa2 in C2C12 cells, we assessed whether Foxa2 can be regulated by PPAR β/δ , the most abundant PPAR isoform in skeletal muscle. C2C12 myotubes were treated with 1 μ M GW501516 (GW), a highly selective PPAR β/δ agonist. The mRNA expression of Foxa2 was increased by two-fold at 24 h and remained elevated at 48 h of treatment with GW (Figure 3.3A). Western blot analysis also revealed a progressive increase in Foxa2 protein at 12 h, peaking at 24 h, and remained upregulated at 72 h of GW treatment (Figure 3.3B).

Figure 3.3 PPAR β/δ agonist increased Foxa2 expression in C2C12 myotubes.

(A) Fold change of Foxa2 mRNA expression in C2C12 myotubes treated with 1 μ M GW501516 over 48 hours. DMSO was used as vehicle control. **(B)** Representative immunoblot (top) and densitometric analysis (bottom) of Foxa2 in myotubes treated with 1 μ M GW501516 treatment over 72 hours. Actin was used as loading control. All data are presented as the mean $\pm$ SEM from 3 independent experiments. *P < 0.05.

To determine if *Foxa2* is transcriptional regulated by PPAR β/δ in skeletal muscle, we performed chromatin immunoprecipitation (ChIP) using PPAR β/δ antibody. In the first instance, we performed in silico analysis of the intragenic regulatory region at the promoter and the first intron of the mouse *Foxa2* gene. We identified two putative PPAR response elements (PPREs) — the first PPRE1 was at position -13bp upstream, and the second PPRE2 resided in the first intron at +2150bp downstream of the *Foxa2* transcription start site (Figure 3.4A). Next, ChIP using PPAR β/δ antibody was performed to confirm its binding. C2C12 were treated with 1 μ M GW for 6 h prior to ChIP analysis. DNA gel electrophoresis analysis of the protein-DNA interactions revealed the binding of PPAR β/δ to PPRE1 (Figure 3.4B), but not PPRE2 of *Foxa2* gene (Figure 3.4C). The interaction of PPAR β/δ on *Foxa2* promoter region was also validated with ChIP-qPCR. Our observation indicated that PPAR β/δ interacted with PPRE1 at basal level, and the occupancy was increased in the presence of 1 μ M GW (Figure 3.4D). To further validate the functionality and specificity of PPAR β/δ binding at PPRE1, we constructed reporter gene vectors containing PPRE1 and mutated PPRE1 (Δ PPRE1) sequences and the relative luciferase activities were detected in HEK293T cells in the presence of increasing GW concentrations. The Δ PPRE1 was designed with two nucleotide substitution at +4 and +5 position of PPRE1 sequence, which PPAR is known to bind at the 5' hexad sequence of consensus PPREs. Firefly luciferase assay revealed the increased expression of luciferase vector containing PPRE1 sequence in the presence of 1 μ M and 10 μ M GW, but not in luciferase vector containing Δ PPRE1 sequence (Figure 3.4E).

In summary, we identified mouse *Foxa2* as a direct target gene of PPAR β/δ in skeletal muscle, whose mRNA and protein expressions are increased by ligand-activated PPAR β/δ .

Figure 3.4 PPAR β/δ binds to the promoter region of mouse *Foxa2* gene.

(A) A schematic diagram of putative PPREs in the promoter and the first intronic region of mouse *Foxa2* gene. The table showed the predicted PPRE sequences and its score, with a maximum score of 1.0 which reflect the predicted confidence using NUBIScan, a prediction software for nuclear receptor response elements. (B) ChIP analysis of PPRE1 with PPAR β/δ antibody or pre-immune IgG (p.i.) in C2C12 treated with either vehicle (Veh) or 1 μ M GW. (C) ChIP analysis of PPRE2 with PPAR β/δ antibody or pre-immune IgG (p.i.) in veh or GW treated C2C12. (D) Quantitative ChIP-qPCR analysis of PPAR β/δ binding at PPRE1 in the presence or absence of GW treatment. (E) Relative luciferase activity in HEK293T cells to screen reporter gene constructs with either PPRE1 sequence or Δ PPRE1 sequence with 2 nucleotide substitution at position +4 and +5, in the presence 1 μ M and 10 μ M GW for 6 h. DMSO was used as the vehicle control. The data are presented as the mean $\pm$ SEM from 3 independent experiments. *P < 0.05; **P < 0.01.

3.4 Foxa2-mediated downstream genes are associated with lipid metabolism, inflammation response, and injury.

To gain further insights into the cellular regulatory processes mediated through Foxa2, we evaluated known downstream targets of Foxa2 using the Ingenuity Pathway Analysis (IPA, Qiagen Inc, USA). Starting with Foxa2 as our molecule of interest, we generated gene connectivity, comprising of known Foxa2-targeted molecules through IPA's Ingenuity Knowledge Base data repository. A total of 223 genes were revealed as Foxa2 direct target genes. The list of Foxa2 target genes was then subjected to gene ontology (GO) analyses. Here, we winnowed our analyses to post-natal functions of Foxa2. The GO analyses revealed the top 5 enriched diseases and biological functions as lipid metabolism, molecular transport, metabolic disease, organismal injury and abnormalities, and inflammatory response (Figure 3.5A). Further interrogation revealed 217 unique molecules in the ontologies of lipid metabolism, organismal injury and abnormalities, and inflammatory responses (Table 3.1). Out of these, 48% (104 molecules) shared overlapping functions, with 30% (65 molecules) exhibit functions from all three ontologies. Notably, 97% (104 molecules) of the lipid metabolism-associated GO subset have overlapping functions in inflammatory responses, organismal injury and abnormalities, or both (Figure 3.5B). The *in silico* analyses of Foxa2 downstream target genes suggest important roles in the regulation of lipid metabolism, inflammation response and injury.

A

B

Figure 3.5 Gene ontologies of Foxa2 downstream genes.

(A) The top 5 gene ontologies (GO) of Foxa2-regulated genes and the constituent number of molecules identified using Ingenuity Pathway Analysis (IPA). A lower P-value indicates a stronger association with the indicated GO. (B) A total of 223 genes are known to be regulated by Foxa2. Out of 223 genes (grey), 213 unique molecules were stratified in respect to lipid metabolism (green), organismal injury and abnormalities (blue) and inflammatory response (orange) ontologies.

Table 3.1 List of known Foxa2-regulated genes and their respective annotated GO.

Gene	Organismal injury and abnormalities	Inflammatory response	Lipid metabolism	Metabolic disease	Molecular Transport
A2M	✓		✓	✓	✓
ABCA3	✓	✓	✓		✓
ABCB1	✓	✓	✓		✓
ABCB4	✓	✓	✓		✓
ABCC8	✓		✓	✓	✓
ACTA2	✓	✓		✓	
ACTC1	✓			✓	
ACTG2	✓				
ADAR	✓				
ADH1C	✓		✓		✓
AFP	✓		✓		✓
AGL	✓				✓
AGR2	✓	✓			
AKAP8	✓				
ALAS1	✓				
ALB	✓	✓	✓	✓	✓
ALDOB	✓				
ALOX15	✓	✓	✓	✓	✓
ALOX5	✓	✓	✓	✓	✓
AMY2B	✓	✓		✓	
APOA1	✓	✓	✓	✓	✓
APOB	✓	✓	✓	✓	✓
APOC3	✓		✓	✓	✓
APOM	✓	✓	✓	✓	✓
ARNT	✓		✓	✓	✓
ASTN1	✓				
BIRC2	✓	✓			
C3	✓	✓	✓	✓	✓
C5	✓	✓	✓	✓	✓
CCL11	✓	✓	✓		✓
CCL17	✓	✓			✓
CCL22	✓	✓	✓	✓	✓
CCL24	✓	✓			✓
Ccl8	✓	✓		✓	
CCND1	✓	✓		✓	
CCR5	✓	✓		✓	✓
CD55	✓	✓		✓	
CDH1	✓	✓			✓
CDX2	✓				✓
CEBPA	✓	✓	✓	✓	✓
CHI3L1	✓	✓		✓	
CHUK	✓	✓		✓	✓
CLCA1	✓	✓			✓
CNN1	✓			✓	
CPT1A	✓		✓	✓	✓
CXCR4	✓	✓		✓	✓
CYP2A6	✓		✓		
CYP2C9	✓	✓	✓	✓	
DACH1	✓				
DCT	✓				
DCX	✓			✓	
DDC	✓			✓	✓

DLK1	✓		✓	✓	✓
EN1	✓				
EN2	✓				
EP300	✓	✓	✓	✓	✓
EPCAM	✓				
EPHX1	✓		✓		✓
EPX	✓	✓			
FABP5	✓	✓	✓	✓	✓
FGB	✓	✓			✓
FGF8	✓		✓		
FOXA1	✓		✓		✓
FOXA3	✓				
FOXN2	✓				
FOXO1	✓	✓	✓	✓	✓
G6PC	✓	✓	✓	✓	✓
G6PC2	✓		✓		✓
GATA4	✓		✓		✓
GATA6	✓		✓	✓	✓
GCG	✓	✓	✓	✓	✓
Gcg	✓	✓	✓	✓	✓
GCK	✓		✓	✓	✓
GFRA4	✓				
GLIS3	✓			✓	✓
GRIN3A	✓	✓		✓	✓
GSC	✓				
GSTM3	✓				
H2AFZ	✓		✓		✓
HADH	✓		✓	✓	✓
HCRT	✓				✓
HES6	✓				
HHIP	✓				
HIF1A	✓	✓	✓	✓	✓
HK2	✓			✓	✓
Hmga2	✓				
HMGCS2	✓	✓	✓	✓	
HNF1A	✓		✓	✓	✓
HNF1B	✓	✓	✓	✓	
HNF4A	✓	✓	✓	✓	✓
HOXA5	✓	✓	✓		✓
HOXB13	✓				
HOXD13	✓				
HPGD	✓	✓	✓	✓	✓
IAPP	✓		✓	✓	✓
IGF1	✓	✓	✓	✓	✓
IGFBP1	✓	✓		✓	✓
IGFBP5	✓	✓		✓	✓
IL13	✓	✓	✓	✓	✓
IL13RA2	✓	✓			
IL33	✓	✓	✓	✓	✓
IL4	✓	✓	✓	✓	✓
IL5	✓	✓	✓		
IL6	✓	✓	✓	✓	✓
Ins1	✓	✓	✓	✓	✓
ISL1	✓	✓			
KAT5	✓		✓		✓

KCNJ11	✓			✓	✓
LHX1	✓				
LIPE	✓	✓	✓	✓	✓
LPL	✓	✓	✓	✓	✓
LYZ	✓	✓		✓	✓
MAFA	✓			✓	✓
MAP1B	✓				✓
mir-122	✓	✓	✓	✓	✓
MLXIPL	✓		✓	✓	✓
Mt1	✓	✓	✓	✓	✓
MTTP	✓	✓	✓	✓	✓
MTUS1	✓				
MUC2	✓	✓			
MUC4	✓				
MUC5AC	✓	✓			
MUC5B	✓	✓			
MUC6	✓				
MYH11	✓			✓	✓
MYL9	✓	✓			
MYOCD	✓			✓	
NACC1	✓				
NAP1L1	✓				
NCOA1	✓		✓		✓
NEUROD1	✓			✓	✓
NEUROG2	✓				
NEUROG3	✓		✓	✓	✓
NFKB2	✓	✓			
NFKBIE	✓				✓
NKX2-1	✓		✓		✓
NKX6-1	✓			✓	
NPTX2	✓				
NR0B2	✓	✓	✓	✓	✓
NR1H4	✓	✓	✓	✓	✓
NR1I2	✓	✓	✓		✓
NR4A2	✓	✓	✓	✓	
NR5A2	✓	✓	✓		✓
NUMB	✓	✓			
ONECUT1	✓		✓	✓	✓
OTX2	✓				
PA2G4	✓				
PAX6	✓	✓		✓	✓
PCK1	✓		✓	✓	✓
PDHX	✓				
PDX1	✓	✓		✓	✓
PKM	✓	✓	✓	✓	✓
PMCH	✓		✓		✓
PNLIPRP1	✓	✓			
PON1	✓	✓	✓	✓	✓
PPARA	✓	✓	✓	✓	✓
PPARG	✓	✓	✓	✓	✓
PPARGC1B	✓		✓	✓	✓
PPP1R14C	✓				
PRKDC	✓		✓	✓	
PROS1	✓	✓		✓	
PTGDS	✓	✓	✓	✓	✓

PYY	✓		✓	✓	✓
RARA	✓	✓	✓		✓
RARB	✓	✓	✓		
RARG	✓	✓	✓		
Retnla	✓	✓			
RNASE2	✓	✓			
RORA	✓	✓	✓	✓	✓
RORC	✓	✓	✓	✓	
SAA1	✓	✓	✓	✓	✓
Saa3	✓	✓			
SALL1	✓				
SATB2	✓				
SCD	✓	✓	✓	✓	✓
SCGB1A1	✓	✓			
SERPINA1	✓	✓	✓	✓	✓
SFTPA1	✓	✓	✓		✓
SFTPB	✓		✓		✓
SFTPD	✓	✓	✓	✓	✓
SH3PXD2A	✓				
SHH	✓	✓			✓
SLC10A1	✓	✓	✓		✓
SLC2A2	✓		✓	✓	✓
SLC2A4	✓	✓	✓	✓	✓
SLC6A15	✓				✓
SLCO1B3	✓		✓	✓	✓
SMARCA4	✓				
SNAI1	✓	✓			
SOD3	✓	✓			
SOX17	✓	✓	✓		✓
SST	✓	✓	✓		✓
ST18	✓				
TACR3	✓				✓
TAGLN	✓			✓	
TAT	✓				
TBX1	✓				
TBXAS1	✓	✓	✓		
TCF7	✓	✓			
TDO2	✓				
TF	✓	✓		✓	✓
TGFB1	✓	✓	✓	✓	✓
TH	✓	✓	✓	✓	✓
THBS3	✓				
TLE1	✓				
TNNI1	✓				
TTR	✓		✓	✓	✓
UCP2	✓		✓	✓	✓
UCP3	✓		✓	✓	✓
UGT2B17	✓		✓		
UST	✓				
WNT7A	✓				✓
WNT7B	✓	✓			
XRCC6	✓	✓			

3.5 Foxa2 deficiency increases pro-inflammatory response in skeletal muscle.

As the downstream targets of Foxa2 displayed common GO annotations in the inflammatory response and organismal injury and abnormalities, we hypothesized that Foxa2 may play a predominant cytoprotective role in skeletal muscle. To validate our IPA analysis, we first studied the effect of reduced Foxa2 in skeletal muscle through shRNA inhibition. The gene expression of Foxa2 in C2C12 myoblast was knocked down by 70% efficiency (Figure 3.6A), validated through RT-qPCR, while immunoblot analyses revealed 60% efficiency knockdown in Foxa2 protein (Figure 2.6B). Next, we analyzed the mRNA levels by RT-qPCR of several Foxa2 downstream genes involved in lipid and glucose metabolism as well as pro-inflammatory genes (Figure 3.6C). We observed that the genes involved in lipid transport and glucose metabolism such as *Cpt1a*, *Lpl*, *Hk2*, *Ppard* were significantly upregulated, albeit marginally. In contrast, a steep increase in the mRNA levels of pro-inflammatory genes such as *Il6* (55-fold) and *Tnfa* (6-fold) was detected.

Foxa2 has been previously described to negatively regulate *Il6* gene expression via direct binding at *Il6* promoter region in various tissues such as liver and epithelial cells in both mouse and humans, but not in skeletal muscle [201,202]. To verify this plausible interaction in skeletal muscle, ChIP analysis was performed the promoter region of *Il6* gene in C2C12 myoblasts. ChIP analyses revealed the interaction of Foxa2 and *Il6* gene, thus confirmed Foxa2 negatively regulates *Il6* expression in skeletal muscle (Figure 3.6D).

Figure 3.6 Loss of Foxa2 induced pro-inflammatory response in skeletal muscle.

(A) Fold change of Foxa2 mRNA expression in Foxa2-knockdown myoblasts. (B) Representative immunoblot (top) and densitometric analysis (bottom) of Foxa2 in Foxa2-knockdown myoblasts. (C) Relative fold change of genes involved in lipid, glucose and skeletal muscle metabolism in Foxa2-knockdown myoblasts. (D) Schematic diagram of Foxa2 binding site at the promoter region of mouse *Il6* gene (left). ChIP analysis of *Il6* promoter region with Foxa2 antibody in C2C12. All data are presented as the mean $\pm$ SEM from 3 independent experiments. * $P < 0.05$; ** $P < 0.01$.

Subsequently, we overexpressed Foxa2 myc-tagged protein in shFoxa2 myoblasts (shFoxa2+OE; Figure 3.7 A, B). shFoxa2+OE myoblasts approximately induced 305-fold change increased in mRNA expression (Figure 3.7A) and 2-fold change increase in protein expression, compared to shFoxa2 myoblasts (Figure 3.7B). Pro-inflammatory myokine expressions such as Tnfa, Il6 and Ccl2 in shFoxa2+OE myoblasts were significantly reduced compared to shFoxa2 myoblasts (Figure 3.7C). Together these results suggest that Foxa2 regulates cytokine expression in skeletal muscle.

Figure 3.7 Rescue of Foxa2-knockdown myoblasts.

(A) Fold change of Foxa2 mRNA expression in transfected C2C12 myoblasts with either Foxa2-myc tagged, shFoxa2 or both vectors. (B) Representative immunoblot of Foxa2 and Myc in transfected C2C12 myoblasts. β -Tubulin was used as loading control. (C) Gene expression of inflammatory cytokines in transfected myoblasts. All data are presented as the mean + SEM from 3 independent experiments. **P < 0.01.

The loss of Foxa2 expression in skeletal muscle increased cytokines expression, we asked if the inverse is true. We examined the effects of lipopolysaccharide (LPS), a classical activator of inflammation in both immune and non-immune tissues, on Foxa2 expression in skeletal muscle. In the presence of 100 ng/mL LPS, Foxa2 mRNA expression was reduced by 65% (Figure 3.8A) while immunoblot analysis showed a 50% reduction of Foxa2 protein expression level in C2C12 myoblasts compared to vehicle-treated control (Figure 3.8B). Similarly, in differentiated myotubes, Foxa2 protein expression was reduced by 30% compared to vehicle (Figure 3.8C). Next, we asked if the ligand activation of PPAR β/δ could increase Foxa2 expression and consequently attenuate the LPS-induced inflammatory response in C2C12. To this end, we treated wild-type and Foxa2-knockdown C2C12 myoblasts with LPS in the presence or absence of PPAR β/δ agonist GW for 24 h. The gene expression of Foxa2 mRNA was partially rescued when cells were treated with GW in the presence of 100 ng/mL LPS (Figure 3.8C). Foxa2 knockdown cells were not affected by either treatment, as expected.

Next, we performed multiplex cytokine array on the conditioned medium of each treatment. The analysis revealed and confirmed the pronounced increased of cytokine expression in shFoxa2 cells, while vehicle-treated C2C12 produced few species of cytokines in low quantity in the milieu (Figure 3.8D). The treatment with LPS alone caused an increase in inflammatory cytokines in vehicle-treated cells, as per reported [203,204]. Consistent with qPCR data, more inflammatory cytokines and chemokines such as Il6, Il-1b and Ccl4 were secreted from shFoxa2 than vehicle-treated cells (Figure 3.8D). We observed a reduction in several cytokine species in the medium of vehicle-treated C2C12 when co-treated with LPS and GW (Figure 3.8D). Co-treatment

of LPS and GW in shFoxa2 cells did not attenuate the inflammatory status, most likely due to the already exaggerated cytokine production.

Overall, these findings show that Foxa2 has cytoprotective role in skeletal muscle against inflammatory response. A reduced Foxa2 expression resulted in an excessive cytokine production that potentially damages tissues.

Figure 3.8 LPS-induced inflammatory response reduced Foxa2 expression in skeletal muscle.

(A) Fold change of Foxa2 mRNA expression in LPS-treated C2C12 myoblasts, compared to vehicle-treated cells. (B) Representative immunoblot of Foxa2 in LPS-treated cells. (C) Relative Foxa2 mRNA expression in wild-type and shFoxa2 cells, treated in the presence of either LPS, GW or both. (D) Cytokine concentrations in culture medium collected from 3 independent experiments. Culture medium was collected from wild-type or shFoxa2 cells treated with LPS, GW, or both, for 24 h. Heatmap showing mean cytokine concentrations. All data are presented as the mean $\pm$ SEM from 3 independent experiments. **P < 0.01; N.D. not detected (grey).

3.6 Foxa2 confers cytoprotective role in skeletal muscle in part via PPAR β/δ

Intramuscular injection of carrageenan, CA, is a well-established system for inducing skeletal muscle inflammation *in vivo* [205,206]. To validate our *in vitro* observations, we triggered a CA-induced inflammation in the TA muscle and examined the effect of PPAR β/δ -mediated upregulation of Foxa2. We injected CA into the muscle of one hindlimb and saline into the contralateral muscle. CA forms bolus that is localized to the site of injection which induced a local inflammation by 6 h. This response by the skeletal muscles to CA was also reflected by an increase in the weights of Quad (Figure 3.89) and TA muscles (Figure 3.9B). We performed qPCR of several proinflammatory cytokines expression which was increased in CA, but not in saline-treated muscle tissues (Figure 3.9C).

Figure 3.9 CA-induced inflammation in mice hindlimb muscle.

(A) Weight of Quad muscles at 6 h after saline or CA treatment on contralateral muscles.
(B) Weight of TA muscles at 6 h treatment with saline or CA on contralateral muscles.
(C) Relative mRNA expression of pro-inflammatory genes in TA muscles at 6h after saline- and CA intramuscular injection. muscles. All data are presented as the mean $\pm$ SEM. *P < 0.05; **P < 0.01. (Mann-Whitney U test; n=5.)

Next, we co-injected GW in hindlimb muscles, in the presence or absence of CA for 6 h. Both Quad and TA muscle weights were taken after 6 h treatment (Figure 3.10A, B). In the absence of GW, CA-mediated inflammation did not alter Foxa2 mRNA expression (Figure 3.10C) but reduced Foxa2 protein expression compared to the vehicle-treated TA muscles (Figure 3.10D). In the presence of GW, both mRNA and protein expression of Foxa2 were increased compared to the vehicle-treated TA muscle (Figure 3.10C, D). No statistically significant differences in Foxa2 was observed between GW alone and GW with CA co-treated muscles.

Figure 3.10 CA-induced inflammation in the presence or absence of GW.

(A) Weight of Quad muscles at 6 h after GW or CA+GW treatment on contralateral muscles. (B) Weight of TA muscles at 6 h after GW or CA+GW treatment on contralateral muscles. (C) Relative Foxa2 mRNA expression in TA muscles at 6h CA-induced inflammation treated in the presence or absence of GW. (Mann-Whitney U test; n=5.) (D) Representative immunoblot and densitometric analysis of Foxa2 protein expression in TA muscles at 6h CA-induced inflammation treated in the presence or absence of GW. (Multiple comparisons one-way ANOVA test; n=5). All data are presented as the mean $\pm$ SEM. *P < 0.05; **P < 0.01; N.S., not significant.

We further assessed if the increased Foxa2 expression by GW could alleviate CA-induced inflammation and damages. We looked at the muscle morphology for pathohistological differences and performed multiplex cytokine array to measure any differences in the cytokine secretion in the various treated mice. As CA-induced inflammatory response at the injected site of muscle, we expected observable tissue damages, secretion of cytokines from the injured muscles, and infiltration of immune cells and other mononucleated cells that initiate skeletal muscle repair and regeneration. The cross-sectional H&E stains of TA muscles showed increased infiltration of mononucleated cells into CA-induced tissue damages than saline treatment (Figure 3.11A). On the other hand, GW co-treatment resulted in less exaggerated tissue damage and the infiltrated mononucleated cells. In concordance to the observed infiltration of mononucleated cells at the injured site, our multiplex cytokine analysis revealed an increased presence of cytokines in CA-induced muscle inflammation (Figure 3.11B). The GW+CA co-treatment resulted in a decrease in most, but not all, species of cytokine/chemokine production.

The current in vivo study has its limitation. We were unable to study the effect of Foxa2 deficiency in skeletal muscle as the whole body-knockout mouse of Foxa2 was embryonic lethal and mouse with muscle-specific deletion Foxa2 was not available. We causally circumvented this limitation by pharmacologically increasing endogenous Foxa2 expression using PPAR β/δ agonist. Together with the various in vitro finding, our study suggests that Foxa2 confers cytoprotection against the damage caused by excessive inflammatory response in skeletal muscle.

Figure 3.11 PPAR β/δ agonist treatment reduced pro-inflammatory cytokines in CA-induced muscle inflammation.

(A) Representative cross-sectional H&E stains of TA muscles at 6 h of CA-induced inflammation (CA only) and in the presence of GW co-treatment (CA + GW), compared to vehicle-treated mice. (B) Cytokine array analysis of Quad muscle lysates after 6 h of CA-induced inflammation (CA) and in the presence of GW co-treatment (CA+GW), compared to vehicle-treated muscle. Heatmap showing mean cytokine concentrations. Data are presented as the mean from 3 independent experiments. N.D. not detected (grey).

3.7 Concluding remarks

Overall, we showed that Foxa2 in skeletal muscle plays a dominant role in modulating inflammatory response than tissue metabolism. Firstly, the expression of Foxa2 in skeletal muscle was affected by inflammatory stimuli rather than hormonal regulation of metabolism, specifically insulin and glucocorticoids. Secondly, we also showed that PPAR β/δ directly regulate Foxa2 in skeletal muscle. Activation of PPAR β/δ using synthetic ligand increased its occupancy on the PPRE in the promoter of the *Foxa2* gene to stimulate the expression of endogenous Foxa2. Third, using IPA analysis and gene connectivity mapping, our interrogation revealed that known Foxa2 downstream target genes were largely involved in the regulation of 2 broad categories of lipid metabolism, and inflammatory response. Fourth, the Foxa2 deficiency in C2C12 cells led to an enhanced cytokine and chemokine production. Finally, in CA-induced muscle inflammation and tissue damage model, we showed that Foxa2 prevented excessive inflammatory response in skeletal muscle and reduce tissue damages. This inflammation-associated tissue damages can be attenuated, at least in part, by ligand activation of nuclear receptor PPAR β/δ .

CHAPTER 4. FUTURE DIRECTION

Involvement of skeletal muscle Foxa2 in pathological conditions

4.1 Introduction

Our previous finding revealed a novel cytoprotective role for Foxa2 in acute inflammation-associated tissue damage. The CA-induced muscle inflammation model mimics exercise-induced inflammation without confounding the damages or other systemic factors that are often associated with exercise-induced injury [207]. The growing realization low-grade chronic systemic inflammation causes and advances many common diseases, such as cardiometabolic health. However, a role for skeletal muscle Foxa2 in systemic inflammation and chronic diseases remains unclear. Moreover, the knockdown of Foxa2 in C2C12 myoblasts induced a plethora secretion of pro-inflammatory cytokines. Skeletal muscle, a secretory endocrine organ which produce cytokines which have autocrine, paracrine and/or endocrine actions involved in inflammatory processes during physiological stimuli and pathological conditions [208,209]. Over the past decades, it has been demonstrated intensity of inflammatory reaction and inflammatory response efficiency play critical roles the myogenic and regenerative program in skeletal muscle, with controlled and efficient inflammatory response is necessary for optimal muscle regeneration [210] while chronic elevated inflammation leads to the onset of muscle wasting [209]. Herein, we explored the possible involvement of Foxa2 in various pathological conditions in skeletal muscle.

4.2 Foxa2 deficiency inhibits skeletal muscle differentiation

Myogenic differentiation in C2C12 is triggered upon serum starvation, as described by Yaffe and Saxel [211], which have been characterized to mimic *in vivo* muscle development and thus representing an excellent model to study myogenesis and response to stimuli [212,213]. Moreover, Foxa2 had been reported to regulate

embryonic development and post-natal maturation of pancreatic beta cells [68,94,202]. Thus, we attempted to differentiate Foxa2 overexpressing and knockdown myoblasts over 5 days (120 h). Phenotypically, Foxa2 overexpressing cells differentiate into thin myotubes with high myosin heavy chain (MyH) expression, similar to control cells (Figure 4.1A). In stark contrast, shFoxa2 cells did not differentiate into myotube and MyH expression was not detected, and a lack of fusion competency was observed. In shFoxa2+OE cells, nascent myotubes were observed with little MyH expression but the phenotype was not completely rescued compared to the control cells (Figure 4.1A).

To further characterize the role of Foxa2 in muscle differentiation, we checked the expression of several skeletal muscle regulatory genes through immunoblot analysis (Figure 4.1B). Similar to the phenotype observation described above, myosin heavy and light chain was not detected in shFoxa2 cells at day 5 of differentiation, thus confirming the lack of myotube formation. The expression of MyoD in shFoxa2 was observed to be higher than control, while no difference was observed in myogenin expression. In Foxa2OE, the expression of myogenin and MyH expression was lower than control, while MyL was not detected in Foxa2OE, suggesting that the Foxa2OE myotubes were not fully matured myotubes (Figure 4.1B). In shFoxa2+OE cells, no MyH and MyL expression was detected through immunoblot analysis despite MyH was observed through immunofluorescence imaging (Figure 4.1B). The immunoblot analyses of MyoD and myogenin in shFoxa2+OE cells were similar to shFoxa2, despite the rescue in Foxa2 expression (Figure 4.1B).

Together, these results suggested a role of Foxa2 in skeletal muscle differentiation. The lack of Foxa2 in skeletal muscle impaired the myoblast-to-myotube

differentiation process. Moreover, a rescue in Foxa2 expression in shFoxa2 cells did not fully revert the phenotype as per wildtype. The impairment of C2C12 differentiation process may be due to the presence of elevated cytokine secretion but could not be fully accounted for the lack of myotube formation in shFoxa2 cells. This suggests a role of Foxa2 skeletal muscle development and maturation, possibly during the signaling regulation of myoblast fusion during myogenesis.

A **B**

Figure 4.1 Foxa2 deficiency impairs C2C12 differentiation

(A) Representative images of phase contrast and immunostaining for DAPI (blue), MyH (green) or merged (DAPI/MyH) performed in C2C12 myotubes expressing Foxa2OE, shFoxA2 or both. Scale bars: 100 μ M. **(B)** Immunoblot analyses of Foxa2, Myc, MyoD, Myogenin, MyH, and MyL in transfected cells. β -Tubulin was used as loading control.

4.3 Foxa2 is a pan-PPAR target gene

All three PPAR isoforms are expressed in skeletal muscle. Although PPAR α and PPAR γ are present in lower levels compared with PPAR β/δ , the physiological and pharmaceutical activation of two other isotypes has been shown to regulate metabolic processes in skeletal muscle as well. We have demonstrated the direct regulation of Foxa2 by PPAR β/δ . Here, we assessed the selectivity of Foxa2 expression by PPAR isoforms in skeletal muscle. We treated C2C12 myotubes with highly potent PPAR α and PPAR γ agonist, 10 μ M WY14643 (WY) and 10 μ M rosiglitazone (ROS), respectively up to 48 h. Both mRNA and protein expression Foxa2 increased in time-dependent manner in the presence of WY or ROS (Figure 4.2). This observation suggests that Foxa2 is a pan-PPAR target gene in skeletal muscle.

Figure 4.2 Foxa2 is a pan-PPAR target gene in skeletal muscle.

(A) Fold change of Foxa2 mRNA expression in C2C12 myotubes treated with PPAR α agonist, WY14643 (WY) over 48 h. (B) Representative immunoblot of Foxa2 in C2C12 myotubes treated with WY for 48h. (C) Fold change of Foxa2 mRNA expression in myotubes treated with PPAR γ agonist, rosiglitazone (ROS) over 48 h. (D) Representative immunoblot of Foxa2 in C2C12 myotubes treated with ROS for 48h. All data are presented as the mean $\pm$ SEM from 3 independent experiments. *P < 0.05; **P < 0.01.

4.4 Systemic inflammation reduces skeletal muscle Foxa2 in skeletal muscle.

The injection of purified LPS via either the intraperitoneal (i.p.) route or the intravenous route in mice leads to systemic activation of the innate immune system. The expression of skeletal muscle Foxa2 in response to systemic LPS challenge remains unknown. We observed a reduction of Foxa2 mRNA level in skeletal muscle by 2 h after LPS i.p. injection (Figure 4.3). Besides skeletal muscle, we also observed decreased Foxa2 mRNA expression in the lungs, but not the liver (Figure 4.3). These observations were consistent with our previous observation of tissue-specific regulation of Foxa2 expression. The mechanism for such tissue-specific regulation and the functional role of Foxa2 in those tissues will require further investigation.

Figure 4.3 Expression of Foxa2 in various organs in LPS-induced systemic inflammation.

Relative fold change of Foxa2 mRNA expression in skeletal muscle, liver, and lungs at 2 h LPS-induced inflammation via i.p. injection, compared to saline treatment. All data are presented as the mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$; N.S., not significant. (Mann-Whitney U test; $n=3$.)

4.5 High-fat diet treatment in mice reduced Foxa2 expression in skeletal muscle

Obesity and obesity-induced insulin resistance have been intricately associated with low-grade chronic inflammation characterized by the secretion of pro-inflammatory cytokines. Here, we assessed the expression of Foxa2 in skeletal muscle in response to high-fat diet (HFD) treatment (Figure 4.4). Wild-type mice were fed HFD for 8 weeks, and glucose tolerance test was performed in precedent to confirm the glucose intolerance state in mice fed with HFD. Consistent with previous inflammatory models, Foxa2 mRNA expression was reduced in skeletal muscle compared with their litter-batch controls in diet-induced insulin resistance (Figure 4.4).

Figure 4.4 High-fat diet reduced Foxa2 expression in mice skeletal muscle.

8 weeks of HFD treatment in wild-type mice reduced Foxa2 mRNA expression in Quad muscle. All data are presented as the mean $\pm$ SEM. *P < 0.05; **P < 0.01 (Mann-Whitney U test; n=5.)

4.6 Concluding remark

The Foxa2 expression is stimulated by ligand activation of PPAR α and PPAR γ . We further showed that Foxa2 expression in skeletal muscle was reduced during systematic inflammation via i.p. LPS injection, and chronic, low-grade inflammation via HFD treatment. Notably, we revealed the downregulation of Foxa2 mRNA exhibited tissue-specific regulation. Systemic inflammation decreased the expression of Foxa2 in skeletal muscle and lung, but not the liver, suggesting a potential role for Foxa2 in response to inflammatory diseases such as sepsis and pneumonia. The expression of Foxa2 in skeletal muscle was reduced in mice fed with HFD, which was in contradiction with our earlier observation that palmitate increased Foxa2 expression in C2C12 cells. Furthermore, whereas palmitate upregulates Foxa2 expression, oleic fatty acid could not. These observations underscored a need for a better understanding of the interactions among different dietary fats, as well as diet-induced inflammation. HFD-induced inflammation has been crucial in the progression of cardiometabolic diseases, such as type 2 diabetes. This opens new possibilities for treatment and therapy by blocking the inflammatory processes.

CHAPTER 5: DISCUSSION

The Foxa2 transcription factor has been long known to exert pivotal roles in various phases of mammalian life, from the embryonic development to maintenance of homeostasis and metabolism in adult. The role of Foxa2 has been characterized in the liver, pancreas, and adipocytes, in which the deficiency or inactivation of Foxa2 often resulted in blunted transcription control of glucose and lipid metabolism [85,88,92]. Moreover, the activity and expression of Foxa2 are subjected to the circulating levels of insulin, glucagon, and glucocorticoids in these metabolic organs, depending on the nutritional status of the organism. However very little is known about Foxa2 in skeletal muscle, despite skeletal muscle being a massive contributor to whole-body metabolism homeostasis. In chapter 3 of this thesis, we investigated the functional role of Foxa2 in skeletal muscle.

5.1 PPAR β/δ upregulates Foxa2 in skeletal muscle.

We showed that Foxa2 is expressed in skeletal muscle, both *in vitro* and *in vivo*, albeit in varying levels in different muscle type. Higher Foxa2 expression was detected in muscle type with higher proportions of fast-twitch type II fibers (e.g., gastrocnemius) compared to muscle type with predominant slow-twitch type I fibers (e.g., soleus). This difference in expression level may suggest a functional division for Foxa2 between fiber types. In C2C12 cells, Foxa2 expression did not change during differentiation from myoblasts to myotubes, suggesting that myogenic regulatory factors did not regulate Foxa2. However, it cannot be ruled out that Foxa2 may participate in regenerative myogenesis or activation of satellite cell following injury. We also found that the expression of skeletal muscle Foxa2 was not susceptible to hormonal regulation of metabolism *in vitro*, as insulin and glucocorticoid stimulation at an increasing

concentration or drug potency did not alter Foxa2 expression levels. Given that skeletal muscle is highly sensitive to insulin- and glucocorticoid-dependent regulation, as well as its importance in whole-body metabolic homeostasis, this was unexpected. Moreover, skeletal muscle Foxa2 expression selectively differed between saturated and unsaturated fatty acid loading. These results thus suggested that skeletal muscle Foxa2 functions differently from the liver, pancreas, and adipocytes.

Foxa2 has been described as a pioneer transcription factor [214] and has been known to engage in autoregulation by binding to its own promoter to enhance transcription [214]. Foxa2 is also known to regulate a network of genes involved in metabolism, primarily in the liver and adipocytes. However, there is no drug known to directly modulate Foxa2 expression or activity. PPARs are ligand-activated transcription factors that similarly plays a pivotal role in energy homeostasis. Thus far, the regulatory relationship between Foxa2 and PPAR has largely correlative. Rosiglitazone, a selective PPAR γ agonist, has been shown to increase Foxa2 expression in INS-1 pancreatic cells [215], while Foxa2 is recognized as binding partners of PPAR α in the liver [99]. For the first time, we demonstrated ligand-activated PPAR β/δ binds to a functional PPRE at the promoter regulatory region of the Foxa2 gene and increased Foxa2 expression.

5.2 Elevated Foxa2 reduces inflammation-associated damages in the skeletal muscle.

Inflammation is a part of the body defense mechanism in response to stimuli or injury. Besides the infiltration of inflammatory cells in damaged muscle, skeletal

muscle also synthesizes endogenous, muscle-derived cytokines (also called myokines) at the site of injury. These myokines coordinate much of the inflammatory-related events and play an active role as regulators of muscle inflammatory and repair response [216]. Exercise-induced myokines are often provided beneficiary effects on muscle regeneration and counteract systemic inflammation and modulate glucose and lipid metabolism [208]. Hence, myokines have been suggested to have anti-inflammatory roles and exert both local and endocrine effects. Paradoxically, very high-intensity exercise bouts are known to trigger systemic inflammation, a subsequent immunodepression and thus a higher risk for infections [217]. For example, IL6-mediated signaling has been associated with myogenesis and beneficial regulation of energy metabolism after exercise. However, it has long-term implications for persistent inflammatory conditions and chronic diseases. Interestingly, bi-directional regulation of IL6 and Foxa2 have been previously reported. In vitro stimulation with recombinant IL6 suppressed Foxa2 expression in a dose-dependent manner via STAT3/NF- κ B signaling [201], while Foxa2 directly binds at the promoter region of IL6, negatively regulates and suppresses IL6 production [202].

Our IPA analysis and gene connectivity mapping revealed a dichotomous role for Foxa2; one was in regulating energy homeostasis, and the other was on the inflammatory response. We allude to the observations that stimuli- and organ-specific regulation of Foxa2 somewhat reflected the division of these roles. Tissue-specific deletion and knockdown of Foxa2 in organs such as liver and adipose tissue often resulted in metabolic disruption in lipid and glucose homeostasis. A systemic LPS-induced inflammation decreased the expression of Foxa2 in skeletal muscle and lung, but not the liver. Locally induced inflammation using CA in mice hindlimb muscles

also led to a decrease in Foxa2 expression. The decrease in Foxa2 expression in the skeletal muscle resulted in excessive inflammatory response and tissue damage. In effect, the inflammatory cytokine secretion by Foxa2-deficient myoblasts was greater than LPS-mediated inflammatory response in wild-type cells. In vivo, PPAR-mediated increased in Foxa2 expression reduced cytokine secretion and mitigated inflammation-associated tissue damage.

PPAR α and PPAR γ have been implicated in the regulation of inflammatory responses [218,219]. Anti-inflammatory effects of PPAR α and PPAR γ on peripheral organs, the central nervous system (CNS) and immune cells have been described. In particular, PPAR α and PPAR γ inhibit the activation of inflammatory gene expression and interfere with several pro-inflammatory transcription factors signaling pathways, such as NF- κ B and STAT3 signaling [220]. Compared to PPAR α and PPAR γ , relatively little is known about the PPAR β/δ role in the regulation of inflammation response [180]. An anti-inflammatory actions PPAR β/δ and its role in inflammatory response have been reported, though mainly in endothelial cells, skin, and macrophage [221-223]. Unlike PPAR α and PPAR γ , no PPAR β/δ drugs have been approved for clinical use. Early reports on PPAR β/δ agonist L783483 was shown to hinder the initiation phase of the inflammatory process and decreased carrageenan-induced paw edema in rats [224]. We showed that the treatment of PPAR β/δ agonist GW501516 to CA-induced acute intramuscular inflammation reduced the infiltration of mononucleated cells and attenuated cytokine secretion at the site of injury in mice skeletal muscle, partially through Foxa2 upregulation.

A cytoprotective role for Foxa2 in the skeletal muscle was rather unexpected due to the contribution of skeletal muscle to whole-body energy homeostasis. Foxa2 in skeletal muscle helps maintain homeostasis, acting as a gatekeeper to maintain key inflammation parameters at the desired level in the face of external and internal perturbations. This balance of pro- and anti-inflammatory cytokines in inflammation-associated muscle damage is crucial for the coordination of muscle repair and regeneration. Excessive inflammation potentially leads to detrimental effects of secondary damage, increased muscle catabolism and the development of muscle fibrosis [216,225]. Since Foxa2 is negatively regulated in the presence of inflammatory agents, and Foxa2 knockdown leads to an excess release of an inflammatory cytokine. The regulatory role of Foxa2 in skeletal muscle was reminiscent to that in the lungs. Tissue-specific deletion of Foxa2 in respiratory epithelial cell aggravated the expression and production of cytokines and chemokines, leading to spontaneous pulmonary inflammation and goblet cell metaplasia [105]. Our data suggest that therapies that promote PPAR-mediated Foxa2 expression potentially curtail muscular inflammation to reduce secondary tissue damages.

5.3 Potential role for Foxa2 in skeletal muscle metabolism

In recent years, the importance of inflammation as the driver of many chronic diseases such as obesity, cardiovascular disease, and cancer maladies has been gaining recognition [93-95]. Inflammation and lipid metabolism are known to exhibit intricate relationship, evoke negative impact on whole-body energy metabolism and influence disease development when either becomes dysregulated [112,226]. Thus far, the majority of our current knowledge on Foxa2 have been largely focussing on its

metabolic functions.

Obesity, a key feature of metabolic syndrome, has been long associated with chronic low-grade inflammatory condition [227,228]. Moreover, the maintenance of whole-body metabolic homeostasis requires a balanced immune response and an integrated network of multiple cell types. *De novo* expression of Foxa2 had been previously reported in adipocytes of genetic and diet-induced rodent models of obesity, in which Foxa2 expression in differentiated adipocytes induced gene expression involved in glucose and fat metabolism [88]. In the same report, the authors also described the absence of *de novo* Foxa2 expression in skeletal muscle. Instead of *de novo* Foxa2 expression, our preliminary data showed a reduction of Foxa2 gene expression from quadricep muscles of 8-weeks HFD treatment in wild-type mice when compared with their litter-batch fed with control diet. Although with our preliminary findings, we are unable to discern Foxa2 regulatory role on lipid metabolism during inflammation, these findings suggest that a metabolic role for Foxa2 may be tissue-specific, such as adipose versus skeletal muscle. We would expect skeletal muscle Foxa2 has an extended role in metabolic diseases such as obesity and T2DM. In addition, the role of skeletal muscle Foxa2 may not be limited to metabolic diseases or exercise-mediated muscle inflammation. Systemic inflammation is usually present as an underlying pathological condition in many neuromuscular disorders and diseases such as sarcopenia, muscular dystrophy and cancer cachexia [229-231]. The involvement of Foxa2 in these diseases is currently unknown. Currently, our study has been limited to mice models, and our understanding of the role of human Foxa2 in skeletal muscle is extremely scarce. Clearly, future investigation on the mechanism underlying the tissue-specific regulation of Foxa2 and its roles will be necessary. The clinical implications

will also be emphasized with a better understanding of the expression and function of skeletal muscle FOXA2 in human.

CHAPTER 6. CONCLUSION

In this thesis, we have characterized the role of Foxa2 in skeletal muscle. Given the involvement of lipid and glucose metabolism of post-natal Foxa2 in liver and adipose tissues, we initially hypothesized that Foxa2 would have similar metabolic role in skeletal muscle. To begin, we validated the expression of Foxa2 in skeletal muscle in C2C12 cells and various mice hindlimb muscles. Foxa2 is expressed in C2C12 myoblast, and no change in Foxa2 expression was observed during the course of C2C12 differentiation. In mice hindlimb muscles, Foxa2 expression is higher in muscles with larger proportion of type II fibers. Unlike liver, however, skeletal muscle Foxa2 is not affected by glucocorticoids and insulin. Interestingly, Foxa2 increased in the presence of saturated fatty acids. Similarly, knockdown of Foxa2 in C2C12 revealed that Foxa2 might contribute lesser roles in the regulation of skeletal muscle energy homeostasis. Through gene connectivity mapping through IPA, we validate the dichotomous role of Foxa2. The deficiency of Foxa2 in C2C12 revealed an increased in the expression of pro-inflammatory genes and were later validated through cytokine profiling assay. We also asked if inflammation would affect Foxa2 expression, and thus subjected C2C12 cells with the pro-inflammatory stimuli LPS. Indeed, Foxa2 expression was affected and reduced after LPS exposure. Localized, acute inflammation in mice hindlimb muscle induced through CA intramuscular injection also exhibited a decreased in Foxa2 protein expression. Together, our results revealed that skeletal muscle Foxa2 plays a dominant role in inflammatory response rather than lipid metabolism. The cytoprotective role of Foxa2 in skeletal muscle thus suggest as potential target during inflammation-associated muscle damage arise from physical activity and/or inflammatory diseases.

Saturated fatty acids and their derivatives are known endogenous PPAR β/δ agonists, we assessed the potential molecular interaction of PPAR β/δ on Foxa2 gene expression. We demonstrated that PPAR β/δ agonist increased the expression of Foxa2 both in vitro and in vivo settings. This increased Foxa2 expression in skeletal muscle is mediated through direct binding of PPAR β/δ at the PPRE region on the promoter region of *Foxa2* gene. Moreover, we also demonstrated that Foxa2 is a pan-PPAR target gene in skeletal muscle. Subsequently, we showed PPAR β/δ agonist treatment reduced pro-inflammatory cytokine production in CA-mediated inflammation in mice, as well as LPS-induced inflammatory response in C2C12. This suggested that PPAR β/δ exerts anti-inflammatory actions in skeletal muscle, in which PPAR β/δ agonist treatment elevates inflammatory response partially through Foxa2-dependent pathway.

PPAR β/δ agonists have long been considered as potential pharmaceutical drugs in the treatment of T2DM, which is associated with chronic low-grade inflammation. As of current writing, there is no known synthetic agonistic or antagonistic compound that specifically targets Foxa2. Homozygous deletion of Foxa2 in mice are embryonically lethal and skeletal muscle-specific Foxa2 knockout mice are not available. Here, we had described PPAR β/δ as a potential drug target to increase Foxa2 expression, which reduces inflammatory response in skeletal muscle and thus potentially contributes towards the treatment of T2DM.

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Review

An aPPARent Functional Consequence in Skeletal Muscle Physiology via Peroxisome Proliferator-Activated Receptors

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Received: 27 March 2018; Accepted: 8 May 2018; Published: 10 May 2018

Abstract: Skeletal muscle comprises 30–40% of the total body mass and plays a central role in energy homeostasis in the body. The deregulation of energy homeostasis is a common underlying characteristic of metabolic syndrome. Over the past decades, peroxisome proliferator-activated receptors (PPARs) have been shown to play critical regulatory roles in skeletal muscle. The three family members of PPAR have overlapping roles that contribute to the myriad of processes in skeletal muscle. This review aims to provide an overview of the functions of different PPAR members in energy homeostasis as well as during skeletal muscle metabolic disorders, with a particular focus on human and relevant mouse model studies.

Keywords: peroxisome proliferator-activated receptor; skeletal muscle; lipid metabolism; insulin resistance; aging; physical exercise; type 2 diabetes; muscle regeneration

1. Skeletal Muscle

Skeletal muscle is the largest metabolic organ in the human body, and it contributes ~40% of the total human body mass in healthy non-obese adults. Beyond its well-recognized role in physical movement and postural stabilization, the importance of skeletal muscle in the whole-body metabolism has been increasingly acknowledged, as it can impact overall health and quality of life [1]. Skeletal muscle is a heterogeneous tissue composed of different fiber types, and it exhibits high metabolic flexibility when adapting to metabolic or energy demands, as well as prevailing conditions and activities. Skeletal muscle can withstand massive and sudden changes, both mechanically and bioenergetically, from rest to rapid contractile activity, because it has effective mechanisms for coping with ATP consumption and re-synthesis. While skeletal muscle is anatomically fixed at birth in mammals, postnatal muscle growth can undergo cellular changes, such as increases in length and girth, and some myofibers can experience changes in contractile activity and humoral factors in response to the nutrient availability [2]. The mammalian skeletal muscle can be classified across a spectrum, according to its contractile and metabolic properties, but it is broadly classified into two categories, namely, slow-twitch type I fibers and fast-twitch type II fibers. Slow-twitch type I fibers are rich in mitochondria and have a higher insulin sensitivity and glucose transporter 4 (GLUT4) expression levels than the fast-twitch type II fibers [3]. The type I fibers are rich in myoglobin, surrounded by many capillaries, and contain relatively abundant intracellular lipid levels for oxidative metabolism. These characteristics support long-duration contractile activities, such as walking

and postural stabilization. In contrast, the fast-twitch type II fibers are large fibers with vast glycogen reserves that support their role in glycolytic metabolism. Type II fibers produce rapid contractions that are used for intense activities, but these fibers are easily fatigued. In mammals, type II muscle fibers can be further categorized into type IIa (fast-twitch oxidative), type IIb (fast-twitch glycolytic), and type IIx (an intermediate type between IIa and IIb). However, type IIb fibers are not detectable in the human skeletal muscle [4]. Muscle fiber type switching and tissue remodeling can occur on demand during exercise or during obesity and metabolic-related diseases. In response to exercise training, the metabolic phenotype of the muscle that is used changes along with the increase in size and strength. At rest, a trained muscle uses more energy from fat and less from carbohydrates than the untrained muscle [5]. Skeletal muscle is the predominant site of the insulin-mediated glucose uptake. The deregulation of skeletal muscle energy homeostasis plays a major role in the pathogenesis of peripheral insulin resistance and type 2 diabetes mellitus (T2DM). T2DM is characterized by chronic hyperglycemia, as a result of inefficient pancreatic beta-cell insulin secretion compensation. T2DM is also characterized by a chronic increase in plasma free fatty acid (FFA) levels and dyslipidemia. Excessive triglyceride accumulation in skeletal muscle, both the intramuscular and intramyocellular deposition, induces lipotoxicity, reduces glucose uptake, and ultimately leads to insulin resistance and T2DM [6]. Physiologically, the deregulation of the metabolic homeostasis in skeletal muscle causes muscle fiber type switching, from the slow-twitch to fast-twitch, as the disease worsens over time [7]. Understanding the changes in skeletal muscle during obesity and T2DM development is thus crucial for elucidating the underlying causes of insulin resistance.

The peroxisome proliferator-activated receptors (PPARs) have emerged as the master regulators of both lipid and glucose homeostasis, and are considered as valuable pharmaceutical targets for treating metabolic dysfunctions and T2DM. PPARs are ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily, and they are activated by a variety of synthetic ligands and endogenous ligands, such as the naturally occurring FFAs and their metabolites, arachidonic acid and eicosanoids. The synthetic ligands of PPARs have been used successfully to treat T2DM and dyslipidemia. Specifically, thiazolidinediones (TZDs), such as rosiglitazone and pioglitazone, are specific PPAR γ activators and are used as insulin sensitizers in order to improve insulin resistance in T2DM patients. Fibrates include fenofibrate, clofibrate, and ciprofibrate, which exhibit a predominant PPAR α activity and induce lipid uptake and oxidation. The PPAR α agonist clofibrate has been used to treat dyslipidemia. Insulin-sensitizing effects can also occur as a consequence of PPAR α and PPAR β/δ activation. Physiologically, the members of the PPAR family also modulate basic processes, such as proliferation, differentiation, and postnatal development [8,9]. In this review, we will focus on the metabolic regulatory roles of PPARs in the skeletal muscle during healthy and diseased states, primarily with studies that have used human and mouse models.

2. Transcription Regulation by PPARs

Three related PPAR members, each encoded by distinct genes, have been identified and designated as PPAR α , PPAR β/δ , and PPAR γ . PPAR γ has two distinct isoforms, PPAR γ 1 and PPAR γ 2. PPAR γ 2 is predominantly expressed in adipose tissue and is 30 amino acids longer than the PPAR γ 1 at the N-terminal [10]. As with most nuclear receptors, PPARs share modular structural characteristics. The N-terminal A/B domains encode the activation function 1 (AF-1), the C-domain consisting of the DNA binding domain (DBD), the D-domain, or the hinge domain that provides structural flexibility, and the E-domain containing the ligand binding domain (LBD) and the ligand-dependent activation function 2 (AF-2). Of the PPAR members, the LBDs of PPAR α and PPAR γ are the most similar in shape and size, whereas the LBD of PPAR β/δ is significantly smaller [11,12]. The differences in amino acid sequences among the PPAR members also indicate that the LBD pocket of PPAR α is more lipophilic than that of the two others. These structural differences among the PPAR LBDs suggest the influences of the structurally distinct ligands with varying binding affinities that contribute to ligand selectivity [13]. All members of PPAR form obligate heterodimers with retinoid X receptors (RXRs) and bind as a complex to the consensus sequences, known as peroxisome proliferator response elements (PPREs), located in the regulatory region of their target genes.

In addition to ligand binding, the activity of PPARs is also affected by post-translational modifications, such as phosphorylation, SUMOylation, and ubiquitination, as well as through regulatory proteins, such as AMP-activated protein kinase (AMPK) and cryptochrome (CRY1). Regulation by insulin and insulin-induced PPAR phosphorylation has been reported to enhance the PPAR transcriptional activity [14]. Post-translational modification by ubiquitination has been shown to be affected by the presence of the PPAR ligand. In the absence of the ligand, PPAR α and PPAR β/δ are poly-ubiquitinated and targeted for subsequent degradation [15,16]. The presence of PPAR γ agonists, on the other hand, enhances the PPAR γ polyubiquitination, which promotes its degradation. The monoSUMOylation of PPAR α and PPAR γ has been reported, in which the transcriptional activities of both PPARs are inhibited [17]. The role of energy metabolism and circadian regulation in skeletal muscle has recently been understood through the modulation of the PPAR protein. Recent studies by Jordan et al. (2017), on skeletal muscle circadian rhythm, have shown that the circadian transcriptional repressors CRY1 and CRY2 function as co-repressors of PPAR β/δ , possibly via an AMPK-dependent signaling pathway [18]. Collectively, the post-translational regulation of the PPAR protein has a direct impact on the cellular metabolism and energy production.

PPARs are diverse regulators that fundamentally regulate the energy metabolism at the transcription level. Each member displays distinct tissue distribution patterns and pharmacological profiles. PPAR α is highly expressed in active metabolic tissues, such as the liver, kidney, heart, and skeletal muscle [19], whereas PPAR γ is expressed in primarily the white and brown adipose tissue, where most of the free fatty acids are deposited [20]. PPAR β/δ is ubiquitously expressed because of its importance in the systemic and basic cellular functions, which include the energy modulation in metabolically active tissues, inflammation, wound healing, and keratinocyte and intestinal cell differentiation [7,9]. The *PPAR δ* gene ablation in mice results in a high embryonic lethality [21], and the PPAR γ -deficient mice exhibit an embryonic lethality by E10 [22]. These findings highlight the importance and complex physiological roles of PPARs. All three of the PPARs are expressed in the skeletal muscle at different amounts, as follows: PPAR β/δ has the highest expression levels, followed by PPAR α and PPAR γ [23–25].

3. Nutrient Sensing by PPARs

Members of the PPAR family modulate metabolic responses through sensing and responding to fluctuations in the nutrient availability. Major dietary constituents, such as fatty acids and carbohydrates, can regulate the gene expression of several metabolic pathways via hormones and PPARs and, in turn, induce their utilization [26]. In a post-prandial state, the availability of metabolic precursors promotes the synthesis of natural PPAR ligands and induces PPAR trans-regulation so as to promote anabolism and storage. Upon nutrient scarcity, PPARs are directly activated by the release of FFAs from lipid reserves, and they stimulate the transcription of genes that are involved in FFA uptake and fatty acid oxidation in the skeletal muscle, as well as glycogenolysis, gluconeogenesis, and ketone body synthesis in the liver, reviewed in [27].

Nutrient intake and energy metabolism are closely associated and are subject to hormonal regulation. Insulin, one of the main hormones that regulates whole-body metabolism, promotes glucose uptake in the metabolically active tissues, such as the liver, fat, and skeletal muscle. During post-prandial state, insulin is secreted from the pancreatic beta cells into the bloodstream in response to increased blood glucose levels. At the peripheral tissues, such as skeletal muscle, insulin binds to the insulin receptors at the plasma membrane in order to trigger the insulin signaling cascade via insulin receptor substrate 1 (IRS1) phosphorylation, protein kinase B (AKT/PKB) activation, and glucose transporter type 4 (GLUT4) translocation to the plasma membrane [28]. These actions promote an extracellular glucose clearance [29]. Skeletal muscle accounts for over 80% of the insulin-dependent glucose uptake [30]. Glucose serves as an immediate source of energy and is subsequently converted into acetyl-coenzyme A (acetyl-CoA), by the pyruvate dehydrogenase complex (PDC). Then, it is channeled into the tricarboxylic acid (TCA) cycle and undergoes oxidative phosphorylation in the mitochondria [31]. In skeletal muscle, the excess glucose is stored as glycogen or used as a precursor for lipid synthesis [31]. As blood glucose levels drop over time, the body transits from a fed to fasted

state, triggering the change from glucose to free fatty acids (FFAs) as the preferred fuel substrates of the skeletal muscle and liver. This dynamic glucose-FFA cycle, also known as the Randle cycle, provides metabolic flexibility and survival adaptation so as to conserve the whole-body glucose supply and is of major quantitative importance in the skeletal muscle, as reviewed in [32,33].

During fasting, both PPAR α and PPAR β/δ are upregulated in the skeletal muscle in rodents [34], but only PPAR β/δ is upregulated in the human skeletal muscle [7,35]. Upon the increased FFA influx, the FFAs are hydrolyzed into acyl-CoA complexes, which are then channeled into the mitochondria by the carnitine palmitoyltransferase I (CPT1) for fatty acid oxidation. One of the key genes that regulates the glucose-FFA shuttle is the pyruvate dehydrogenase kinase (PDK), which is a classical PPAR target gene. PDK inactivates PDC, via phosphorylation, and reduces oxidation of the glycolysis-derived pyruvate. These effects decrease the glucose utilization in order to conserve glucose. In human skeletal muscle, all four of the PDK isozymes are PPAR β/δ target genes, and PDK2 and PDK4 are the most abundantly expressed [35,36]. In the skeletal muscle of PPAR β/δ knockout mice, PDK4 expression is markedly blunted [37]. Interestingly, the PDK4 expression is unaffected in the skeletal muscle of the fasted PPAR α knockout mice [25]. These findings thus suggest that PPAR β/δ is the primary PPAR member that regulates the skeletal muscle substrate utilization.

4. Regulation of Lipid Metabolism in Skeletal Muscle by PPARs

Fat and excess calories from the diet are converted into the concentrated form of triglycerides to store metabolic energy over extended periods of time. Triglycerides are typically stored in three main organs (ranked in order, from the greatest to least amount stored), namely, adipose tissue, skeletal muscle, and liver [38]. During fasting or increased energy demands, triglycerides in adipose tissue are hydrolyzed into FFAs and delivered to tissues through the action of lipoprotein lipase (LPL), and can be used either for fatty acid β -oxidation in the energy-converting mitochondria or as building blocks for cellular functions and signaling.

Over the past decade of research, PPARs have emerged as master regulators of the lipid metabolism. In humans, skeletal muscle accounts for more than 30% of the total energy expenditure, and up to 70% of this energy is derived from FFAs in resting muscle. Of the three PPAR members, PPAR α and PPAR β/δ play central roles in regulating lipid homeostasis [25]. PPAR γ promotes glucose uptake in skeletal muscle, in order to play a role in insulin-stimulated glucose metabolism [39]. In vivo, PPAR α and PPAR β/δ regulate the genes that are involved in FFA uptake, such as cluster of differentiation 36/SR-B2 (CD36) and LPL; FFA intracellular transport, such as fatty acid binding protein 3 (FABP3); and fatty acid oxidation, such as CPT1 and stearoyl-CoA desaturase (SCD). The genes that are involved in fatty acid oxidation and utilization are similarly regulated by PPAR α and PPAR β/δ in skeletal muscle, as shown by overexpression studies [40–42]. Skeletal muscle-specific PPAR β/δ overexpression also induces characteristic shifts towards oxidative fibers and increased oxidative capacity [7]. Conversely, selective PPAR β/δ ablation in skeletal muscle leads to lower oxidative capacity in the fibers, resulting in obesity and T2DM [43]. In contrast to PPAR β/δ , PPAR α overexpression promotes fiber type shifts towards glycolytic type II fibers, and these fibers are protected from diet-induced obesity. Interestingly, when fed a high-fat diet, PPAR α -overexpressing mice have significantly higher intramuscular triglyceride concentrations than control mice, and they develop glucose intolerance [41]. In PPAR α knockout mice, however, fatty acid oxidation is reduced during starvation despite an increase in oxidative fibers [25].

4.1. Regulation of Lipid Transport in Skeletal Muscle by PPARs

Unlike glucose, which is water soluble, circulating FFAs are usually associated with albumin or exist as fatty esters and phospholipids in lipoproteins. On the plasma membrane surface, LPL mediates the hydrolysis of triglyceride-rich lipoproteins. This hydrolysis releases the encapsulated lipids and is thus considered a rate-limiting step for lipid uptake. The cellular lipid uptake was initially thought to occur via passive diffusion because of the hydrophobic nature of the plasma membrane. However, it is now widely recognized that FFA uptake requires a highly regulated, protein-mediated action

by the transporter proteins. In humans and rodents, CD36, FABPs, and fatty acid transport proteins (FATPs) are co-expressed in the skeletal muscle, which is key in facilitating FA transport, and their expression levels are regulated predominantly by PPAR β/δ [7] (Figure 1). Approximately 70% of total FFA uptake is mediated by CD36 [44], although the mechanisms of FFA transmembrane movement and the binding specificity of CD36 are not understood [45]. It has been suggested that CD36 promotes fatty acid partitioning at the outer leaflet for translocation through the lipid bilayer and that it provides a docking site for FABPs and other enzymes at the intracellular side of the membrane, so as to facilitate the transport of the incoming FFAs [45]. Cytoplasmic FABP (FABPc) serves as an acceptor for FFAs, shuttles them through the cellular compartments, and protects against lipotoxic accumulation and aggregation within the cell [46]. The fatty acid transporters in skeletal muscle exhibit different capacities for FFA transport and metabolism. An in vivo study of CD36, plasma membrane FABP (FABPpm), FATP1 or FATP4 overexpression in the anterior tibialis muscle of rats showed the differential effects on FFA transport and utilization in skeletal muscle [47]. The authors have reported that CD36 and FATP4 are quantitatively the most effective in FFA transport. Interestingly, the transporter overexpression did not alter the rates of FFA esterification into triglycerides, but it increased fatty acid oxidation that was observed with CD36 and FABPpm overexpression [47,48] (Figure 1).

Figure 1. Schematic diagram of skeletal muscle fiber and its fatty acid handling. **(A)** The fate of free fatty acid (FFA) in skeletal muscle. FFA uptake is mediated by receptors, such as CD36, at the plasma membrane. Within the cell, FFA is transported throughout the cellular compartments, via the lipid transporter, FABPc. FFAs can either be targeted to the lipid droplet for storage, translocated to the mitochondria for fatty acid oxidation, or serve as a ligand for peroxisome proliferator-activated receptors (PPARs) within the nucleus. In the skeletal muscle, PPAR α and PPAR β/δ are mainly involved in lipid metabolism regulation. PPAR β/δ is also involved in regulating mitochondria biogenesis while PPAR γ is involved in skeletal muscle insulin sensitivity and glucose regulation. **(B)** The spectrum of skeletal muscle fiber type characteristics. All three of the PPAR isotypes are expressed regardless of the fiber types. Slow-twitch type I fibers are smaller in fiber diameter, with high oxidative capacity and mitochondria density, while fast-twitch type II fibers have a range in their fiber diameters, typically higher glycolytic capacity with lower mitochondria density, and oxidative capacity in comparison to type I fibers. **(C)** Schematic diagram of PPAR protein structure. PPARs are regulated by post-translational modifications, such as phosphorylation, SUMOylation, and ubiquitination in the presence or absence of ligand. Activation function, AF; DNA-binding domain, DBD; Hinge domain, HD; ligand binding domain, LBD.

Mammalian FABPs are small skeletal muscle proteins of approximately 15 kDa and are involved in the reversible binding of FFAs, in order to facilitate trafficking to various cellular compartments, such as peroxisomes, mitochondria, and nuclei. In humans, there are nine FABP isoforms (FABP1-9) that are differentially expressed in metabolically active tissues [49]. In adult skeletal muscle, FABP3 is predominantly expressed and is more abundant in type 1 oxidative fibers than in type 2 glycolytic fibers. FABP3 is responsible for FFA shuttling to the outer mitochondrial membrane, where FFAs are converted into their acyl-CoA derivatives by acyl-CoA synthetase, and are subsequently channeled for mitochondrial β -oxidation [50,51]. A small amount of acyl-CoA is converted into lipid intermediates, such as ceramide, diacylglycerol, and phospholipids, which can function as lipid secondary messengers or modulate membrane structures [52,53]. FABPs have been shown to interact with PPARs in the nucleus, so as to mediate transcriptional activities. Recently, the ligand-sensitive nuclear localization signal in FABP5 protein conformation has been described. In this conformation, FABP5 facilitates PPAR β/δ transcriptional activation through nuclear trafficking of linoleic acid and arachidonic acid [54]. Similar reports have shown that FABP1, FABP2, and FABP3 can increase FFA shuttling to the nucleus and enhance ligand-mediated PPAR α transactivation [51,55,56], whereas PPAR γ agonists can induce the nuclear localization of FABP4 [57,58]. However, the specificity of the lipid species with FABP chaperone activity and the significant impact of each FABP isoform on the transcriptional regulation in the skeletal muscle remains unclear.

4.2. Regulation of Muscle Lipolysis by PPARs

Lipolysis is the process through which FFAs are sequentially hydrolyzed. Lipolysis is first mediated by the rate-limiting enzyme adipose triglyceride lipase (ATGL), which hydrolyzes triglyceride to diacylglycerol and one fatty acid molecule. Diacylglycerol is then converted into monoacylglycerol, by hormone-sensitive lipase (HSL). The final step of FFA hydrolysis by monoacylglycerol lipase (MGL) produces glycerol and the third fatty acid molecule. In the mouse skeletal muscle, lipolysis can occur without stimulation (basal conditions) or with hormone stimulation [59]. Under either condition, ATGL and HSL collectively account for most of the hydrolysis activity [60]. ATGL is an evolutionarily conserved enzyme for fat storage lipolysis [61]. It is highly expressed in type I fibers in both mice and humans and is a reported transcriptional target of PPAR α in rodents [62]. ATGL knockout mice have a shorter life-span and exhibit defective lipolysis and increased triglyceride accumulation in non-adipose tissues, including skeletal muscle [63,64]. These mice also show a concomitant decrease in muscle relaxation and have an increased reliance on carbohydrates as the major fuel source at rest [65]. Interestingly, pharmacological treatment of ATGL knockout mice with PPAR α agonists reversed the excessive systemic lipid accumulation, improved metabolic flexibility in substrate switching from glucose to fatty acids, and prevented premature death [64]. ATGL overexpression in different muscles has varied effects on skeletal muscle fatty acid oxidation in mice. These varied effects are most likely due to the differential expression levels of ATGL among fiber types. Increased fatty acid oxidation was reported upon ATGL overexpression in the soleus muscle via electroporation [62]. However, adenovirus-mediated ATGL overexpression in the tibialis anterior muscle was not sufficient to alter fatty acid oxidation rates [66]. Similarly, mutations in the *PNPLA2* gene, which encodes ATGL in humans, can lead to neutral lipid storage diseases with myopathy. In humans, ATGL is exclusively expressed in type I muscle fibers and plays an important role in skeletal muscle FA turnover [67]. ATGL deficiency in young adults resulted in increased lipid accumulation in primarily type I skeletal muscle [68]. However, treatment with a PPAR α agonist was less successful in humans than in rodents [69].

4.3. Regulation of Muscle Lipid Storage by PPARs

Skeletal muscles stockpile excess FFAs in lipid droplets as an energy reservoir. These FFA stores are commonly referred to as intramuscular triglycerides. Excess FFAs are converted in the endoplasmic reticulum (ER) and stored in lipid droplets (LDs), which are surrounded by a phospholipid monolayer and LD-associated surface proteins. These LDs are dynamic structures that function as more than

temporary fuel storage. In fact, they serve as a reserve pool of intracellular signaling mediators for ligands, such as PPAR, and are thought to have a protective mechanism against possible lipid aggregation that leads to lipotoxicity and ER stress after the excess uptake of FFAs and sterols. In skeletal muscle, lipid droplets are distributed between myofibrils (intermyofibrillar LDs) and beneath the plasma membrane (subsarcolemmal LDs). These LDs serve as transport organelles between cellular compartments and as a readily available energy pool for short-term or long-term muscular contractions. PPAR agonists have been reported to regulate LD-associated proteins, such as perilipins (PLIN1-5), in various organs. Perilipins, except PLIN1, are expressed in skeletal muscle in humans and rodents [70]. PLIN2, one of the most abundantly expressed LD-coating proteins in skeletal muscle, is thought to maintain insulin sensitivity in skeletal muscle and promote the storage of FFAs in the form of triglycerides [71]. PLIN2 is induced upon PPAR β/δ activation by GW501516 in both human primary myocytes and mouse skeletal muscle [72,73]. In PPAR α knockout mice, PLIN2 and PLIN5 expression levels are decreased in the soleus, whereas PLIN3 and PLIN4 expression levels seem to be unaffected [74]. Interestingly, immunofluorescent staining of human and rodent skeletal muscle sections have shown that PLIN2 is abundantly expressed in type I fibers, which contain more intramuscular triglyceride contents than type II fibers [75,76]. Similarly, the direct regulation of PLIN5 by PPAR β/δ in the soleus and gastrocnemius of wild-type mice has been observed. In this study, a conserved PPRE in humans and mice had been found in the first intron of PLIN5 [74]. However, PLIN5 protein levels in the skeletal muscle did not seem to be altered in PPAR β/δ knockout mice.

PLIN5 has been suggested to regulate FFAs storage and to be involved in skeletal muscle adaptation in type II fibers, in response to exercise and fasting [77]. Similarly to PLIN2, the PLIN5 expression levels are higher in the oxidative fibers than in glycolytic fibers [78], and its protein levels are associated with intramuscular triglyceride levels in both rodents and humans [75]. In glucose-intolerant human subjects, it has been reported that PPAR γ agonists can induce PLIN5 mRNA expression, and PLIN5 mRNA expression is negatively correlated with the body mass index (BMI) in non-diabetic subjects [79]. The role of PPAR regulation and its effects on perilipin functions in skeletal muscle physiology, however, need further investigation, as most of the studies on PLIN5 have been performed in vitro [46].

5. Regulation of Mitochondrial Biogenesis and Function by PPARs

The members of the PPAR γ -coactivator 1 (PGC-1) family, such as PGC-1 α and PGC-1 β , regulate mitochondrial oxidative metabolism and biogenesis, and activate gene transcription through coordination with PPAR α , PPAR β/δ , and other nuclear receptors. PGC-1 α is reported to be a direct target of PPAR β/δ , but not PPAR α , in the skeletal muscle, via agonism [43,80] and during conditions of increased energy demands, such as cold, exercise, and fasting [43,81]. Moreover, in vivo PPAR β/δ overexpression, via electroporation in adult rat muscle, caused an increase in PGC-1 α protein levels [82]. PGC-1 α thus mediates a positive feed-forward transcriptional control of the PPAR-regulated genes that are involved in fatty acid oxidation and carbohydrate metabolism, as well as an auto-regulatory loop, in which PGC-1 α regulates its own gene expression [83]. Gene manipulation of PGC-1 α and PGC-1 β in skeletal muscle produces phenotypes similar to those of PPAR β/δ transgenic mice. Conversely, PGC-1 α or PGC-1 β overexpression in mouse skeletal muscle induces oxidative fiber development, promotes fatty acid oxidation and increases the capacity to sustain physical activity in mice [84,85]. However, PPAR β/δ overexpression in mice does not increase PGC-1 α mRNA levels and does not affect mitochondrial function [42,86]. Additionally, transcription factors such as mitochondrial transcription factor A (TFAM) and mitochondrial transcription factors B1 (TFB1M) and B2 (TFB2M), which directly regulate mitochondrial biogenesis via nuclear respiratory factors (NRF1 and NRF2) are not known to be classic PPAR β/δ target genes [87]. Thus, the precise regulation of PPAR β/δ and PGC-1 α in mitochondrial function and biogenesis has been a long-standing question. Recently, Koh et al. [88] used an electroporation-mediated PPAR β/δ overexpression in mouse muscles to demonstrate that PPAR β/δ modulates mitochondrial biogenesis and PGC-1 α expression, in both a transcriptional manner and a posttranslational manner. PPAR β/δ overexpression in adult mice increases NRF1

and mitochondrial electron transport chain enzyme protein levels, before increasing PGC-1 α protein levels. Moreover, PPAR β/δ decreased PGC-1 α degradation via ubiquitin-proteasome system, through binding and blocking its ubiquitin-binding site. These actions led to the gradual accumulation of the PGC-1 α protein [88]. The authors also reported the auto-regulation of PPAR β/δ , suggesting a feed-forward mechanism that is important in the mitochondrial oxidative metabolism and biogenesis.

6. Dysregulation of Lipid Metabolism and PPAR during Insulin Resistance and T2DM

Insulin resistance is the key pathophysiological feature of obesity and T2DM, and is caused by imbalances in insulin action in peripheral tissues, insulin secretion, or both. In skeletal muscle, the major causes of insulin resistance are thought to be the excess accumulation of intramyocellular lipid (IMCL) and the inhibition of one or several steps in the insulin signaling cascade [89]. IMCL includes all types of lipids within the myocytes. Myocytes are composed of mostly triglycerides, but also include the lipid intermediates of lipid metabolism, ceramides, diacylglycerol, phospholipids, and sphingolipids [90]. The most common cause of lipid accumulation is overnutrition, which leads to an increase in FFA uptake that exceeds the rates of fatty acid oxidation and storage [91]. High IMCL concentrations have also been negatively associated with insulin sensitivity in non-obese adults [92], high-fat diet rodent models [93], and lean offspring of T2DM patients [94]. Similarly, acute lipid overload in skeletal muscle decreases peripheral insulin sensitivity in healthy individuals [5,95]. Paradoxically, it has been reported that endurance athletes are highly insulin-sensitive, despite possessing higher IMCL concentrations than normal healthy individuals. This phenomenon is thus called the ‘athlete’s paradox’ [96]. These trained athletes, however, have a high capacity for fat oxidation and have high glucose disposal rates, but are not totally immune to lipid-induced insulin resistance [5,96]. Unlike obese individuals and T2DM patients, the turnover rates of IMCL in trained athletes is high, and this turnover is an adaptive physiological response rather than a pathological condition [5]. Thus, endurance athletes do not bear the ascribed toxic effects on insulin signaling.

Ceramide and diacylglycerol accumulation interfere with the insulin signaling cascade through the direct interaction with and activation of protein kinase C (PKC) isoforms, so as to reduce glucose uptake [91,97,98]. In the skeletal muscle, a 50% increase in endogenous ceramide levels, induced by treatment with a high concentration of saturated FFAs, is sufficient to inhibit AKT/PKB activity [99]. In obese insulin-resistant human subjects, ceramide concentrations were found to be nearly two-fold higher in muscle compared with lean insulin-sensitive human subjects [100]. In contrast, overexpressing acid ceramidase, which converts ceramide into sphingosine, fully negates the inhibitory effects of high FFA treatment on insulin signaling [101]. Additionally, ceramide has also been shown to stimulate protein phosphatase 2A (PP2A), a phosphatase long known to negatively regulate AKT/PKB [102]. The inverse relationship between ceramide and insulin sensitivity has been reviewed [97]. Furthermore, PKC θ activation by diacylglycerol, induces insulin resistance through inhibiting IRS1-associated phosphatidylinositol-3 kinase (PI3K) activity [103,104]. Diacylglycerol acyltransferase 1 (DGAT1), a downstream PPAR β/δ target gene, catalyzes the conversion of diacylglycerol and fatty acyl-CoA to triglyceride [105]. The skeletal muscle-specific DGAT1-overexpressing mice have low diacylglycerol concentrations and are protected from diet-induced insulin resistance, despite the increased FFAs accumulation in their skeletal muscle [106].

PPAR agonists have been of clinical interest since the discovery of fibrates and the TZDs for treating metabolic-related diseases. Below, we describe the impact of PPAR regulation in skeletal muscle, during insulin resistance and T2DM.

6.1. PPAR γ Agonists and Insulin Resistance and T2DM Treatment

PPAR γ ligands, including TZDs, have hypoglycemic effects, reduce insulin resistance, and improve insulin sensitivity. In the early 1980s, TZDs were reported as insulin sensitizers. Currently, pioglitazone is the only FDA-approved TZD for treating T2DM. This drug has lipid-modifying benefits and can reduce adverse cardiovascular outcomes. The insulin-sensitizing effects of TZDs can be attributed to the

activation of skeletal muscle PPAR γ . This activation maintains insulin signaling activity, even though PPAR γ is expressed at low levels. Given the whole-body skeletal muscle mass, the regulation of the skeletal muscle PPAR γ remains physiologically relevant. The direct action of TZDs on non-adipose tissues has been indicated in adipose tissue-specific PPAR γ -silenced mice, in which TZD treatment improved insulin sensitivity in the skeletal muscle and the liver, despite an increase in triglyceride deposition [107]. In obese Zucker rats, short-term treatment with rosiglitazone increases the skeletal muscle tyrosine phosphorylation of insulin receptor and IRS-1, and induces AKT/PKB activation [108]. Similarly, muscle biopsies that were obtained from T2DM patients that were treated with either rosiglitazone or pioglitazone showed increased insulin-stimulated IRS-1 tyrosine phosphorylation, IRS-1-associated PI3-kinase activity, and AKT/PKB activity [109,110]. The TZD administration, however, has been reported to stimulate skeletal muscle glucose uptake acutely and improve glucose handling through a PPAR γ -independent mechanism [111,112]. Moreover, the PPAR γ -sparing TZD analogs have similar insulin-sensitizing pharmacological effects to rosiglitazone and pioglitazone in rodent models [113]. These results suggest that the insulin-sensitizing effects of TZDs may be independent of PPAR γ regulation, to some degree. Despite the varied pharmacological actions of TZDs via PPAR γ regulation, the role of PPAR γ in the skeletal muscle in glucose homeostasis and insulin sensitivity remains physiologically and clinically relevant. In the human skeletal muscle, PPAR γ expression is acutely regulated and increased by insulin [114]. PPAR γ activation directly regulates the expression of the glucose transporters GLUT1 and GLUT4, and promotes their translocation to the cell surface so as to increase the cellular glucose uptake. In addition, GLUT4 regulation by PPAR γ is remarkably conserved across the vertebrate evolution, from fish to mammals [115]. In L6 muscle cells, PPAR γ agonists, but not PPAR α agonist WY14643, have been shown to increase IRS1 protein expression directly [116]. Moreover, constitutive PPAR γ activation in the mouse skeletal muscle decreases intramuscular lipid accumulation, induces a shift towards the oxidative fiber type, and protects against susceptibility to diet-induced insulin resistance [117]. Conversely, skeletal muscle-specific PPAR γ knockout mice have an increased adiposity and are glucose intolerant and insulin resistant [118,119]. However, the young skeletal muscle of PPAR γ -deficient mice remained responsive to the TZD treatment, despite a high-fat diet-induced hepatic insulin resistance and excess adiposity [119]. These findings led to the suggestion of age-dependent differences in TZD insulin-sensitizing effects and the potential role of tissue crosstalk in the regulation of whole-body insulin sensitivity [120]. In humans, dominant negative PPAR γ mutations are associated with obesity [121], dyslipidemia, and severe insulin resistance [122], whereas a common polymorphism (Pro12Ala) has been shown to decrease PPAR γ receptor activity, improve insulin sensitivity, and decrease T2DM risk [123,124].

6.2. PPAR α Agonists and Insulin Resistance and T2DM Treatment

PPAR α plays a pivotal role in the liver during the nutritional transitions and intricately controls hepatic lipid metabolism and whole-body glucose homeostasis [27]. The role of skeletal muscle PPAR α in regulating the insulin signaling pathway is, however, less clear. Though PPAR α has metabolic regulatory roles, its expression in skeletal muscle remains unchanged during fasting [35]. The clinical use of fibrates for treating hyperlipidemia in obese individuals and T2DM patients was first approved in the late 1960s [125]. The fibrates that are commonly used for clinical treatment are bezafibrate, fenofibrate, and gemfibrozil. Fenofibrate treatment in patients with metabolic syndrome improves lipid profiles and increases insulin sensitivity [126,127]. Recently, bezafibrate has been reported to increase skeletal muscle AKT/PKB phosphorylation and improve the insulin sensitivity in insulin-deficient streptozotocin-treated mice [128]. However, bezafibrate and fenofibrate exhibit weak PPAR β/δ and/or PPAR γ agonist activity [125,129]. Therefore, the direct pharmacological activity of PPAR α on human skeletal muscle insulin sensitivity requires further investigation.

6.3. Evidence for PPAR β/δ Agonist Treatment of Insulin Resistance and T2DM

PPAR β/δ agonists may be insulin sensitizers and have been suggested as a therapeutic approach for treating metabolic dysfunction and T2DM. Currently, there are no PPAR β/δ agonists that are approved for clinical treatment, but several are in the development and clinical study phases [8]. One prominent PPAR β/δ -selective agonist is seladelpar (MBX-8025), which is currently in clinical phase 2/3 for primary biliary cirrhosis, and has previously been shown to improve the insulin sensitivity and dyslipidemia in overweight subjects [130]. The well-known GW501516, though its development was halted in 2007, has since served as an important PPAR β/δ -specific agonist in the elucidation for PPAR β/δ physiological and pathophysiological functions. In animal models of obesity and T2DM, PPAR β/δ activation, through specific agonists or genetic manipulation, ameliorates hyperglycemia, insulin resistance, and dyslipidemia. PPAR β/δ silencing renders mice glucose intolerant and less metabolically active [131]. Similarly, the skeletal muscle-specific PPAR β/δ knockout mice exhibit insulin insensitivity and impaired glucose tolerance [43]. PPAR β/δ agonist treatment improves whole-body insulin sensitivity through complementary actions in the liver and skeletal muscle. In insulin-resistant ob/ob mice, activating PPAR β/δ through GW501516 ameliorates hyperglycemia-mediated glycolysis, and lipogenesis increases in the liver so as to reduce hepatic glucose output. Simultaneously, GW501516 promotes FAO in the skeletal muscle to enhance insulin sensitivity [131]. In addition, long-term GW501516 treatment in wild-type mice reduces body weight and circulating triglyceride levels [42].

7. Regulation of PPARs during Physical Exercise

Adopting and maintaining physical activity is by far the best intervention and prevention for obesity and T2DM. Short-term aerobic exercise can increase glucose uptake by muscles during exercise and can increase insulin-mediated glucose storage in muscles after exercise [132]. In addition, both short-term exercise and endurance training have been reported to increase PPAR β/δ expression levels in both human and rodent muscles [73,133]. In obese and overweight humans, PPAR β/δ expression levels increase with exercise and are associated with the transcription of oxidative and lipoprotein metabolism genes, as well as PGC-1 α [133] (Figure 2). In mice, endogenous PPAR β/δ activation with GW501516 treatment can enhance physical performance and upregulate oxidative genes, mitochondrial biogenesis, and fiber type switching [42]. A recent study showed that GW501516 promotes running endurance by preserving glucose. Activation of muscle PPAR β/δ coordinately reduces glucose catabolism to prevent hypoglycemia and facilitate a progressively longer running time [105]. Similarly, the authors also showed that overexpressing constitutively active PPAR β/δ in rodent skeletal muscle increased the running endurance of these transgenic mice [42]. Furthermore, in the mouse model of ischemic cardiomyopathy, the impaired exercise endurance following myocardial infarction could be reversed by the PPAR β/δ agonist GW501516 [134]. The pharmaceutical activation of PPAR β/δ has attracted much interest as an exercise mimetic to promote oxidative myofibers and running endurance without exercise. Despite a lack of evidence for its clinical safety, GW501516 has become an interest in endurance athletes because of its ability to influence energy expenditure and improve adaptations to training. Unfortunately, this drug has added complexity to the doping dilemma in competitive sports, which has culminated in the suspension of many athletes from the Olympics. The clinical development of PPAR β/δ agonists has been unsuccessful to date, and GW501516 remains a banned metabolic modulator by the World Anti-Doping Agency. Pharmacoequivalents with better safety profiles, however, are still heavily researched [135,136].

Similarly to PPAR β/δ in humans, the expression levels of PPAR α and its downstream target genes increase upon endurance training [133,137]. In skeletal muscle biopsies from spinal cord-injured subjects, the fiber type switching from type I oxidative fibers to type II glycolytic fibers often occurs as a result of muscle disuse, and PPAR α expression is reduced [138]. In rodents, PPAR α knockout mice are less tolerant of endurance exercise, although their skeletal muscle glycogen depletion rate is similar to their wild-type counterparts [25]. Interestingly, genetic variations in PPAR α and PPAR γ appear to

play a role in athleticism. A recent study has found that *PPARA* gene intron 7 G/C polymorphism correlates to an endurance ability. Athletes with high levels of performance in endurance sports have a higher frequency of the GG genotype and G allele [139]. This genotype has also been associated with an increased skeletal muscle fatty acid β -oxidation rate and an increased proportion of type I slow-twitch fibers [140]. The *PPARG* Pro12Ala polymorphism, which is associated with an improved glucose utilization in skeletal muscles, is prevalent in Polish athletes who are involved in sports that involve short-term and intense exercises, such as power-lifters, weight-lifters, and throwers [141].

Figure 2. List of genes regulated by PPAR α (red box), PPAR β/δ (blue box), and PPAR γ (green box) in skeletal muscle. During obesity (red arrows), increased free fatty acid flux leads to excess lipid droplet accumulation, lipid dysregulation, and deregulation of insulin signaling and glucose uptake. Physical exercise can prevent obesity-related disorders and T2DM. Fibroblast growth factor 21, FGF21; malonyl-CoA decarboxylase, MCD; uncoupling protein 1, UCP1; insulin receptor, INSR; hexokinase 2, HK2; and phosphoenolpyruvate, PEPCK.

8. Regulation of Skeletal Muscle Regeneration by PPARs

Skeletal muscle injuries are among the most common soft tissue injuries [142,143], which occur not only during sports traumas and daily activities, but they are also a major concern of diabetic complications, such as muscle ischemia and peripheral vascular disease—the major risk factor of limb amputation in diabetic patients [144,145].

Skeletal muscle regeneration is initiated shortly upon injury and undergoes three main coordinated phases of healing—destruct, repair, and remodel [142]. Upon injury, ruptured myofibers first undergo necrosis, which induces an inflammatory reaction. The damaged tissues are then cleared by infiltrated immune cells, such as macrophages and neutrophils, through phagocytosis [146]. The activation and infiltration of the immune cells further promote the activation of myogenic-reserve stem cells (satellite cells), which then proliferate and differentiate to form new myofibers that orchestrate the muscle reparation [147–149]. During the remodeling phase, angiogenesis of skeletal muscle capillaries and the maturation of regenerated myofibers occur, restoring muscle metabolism and contraction functions [149–152].

8.1. Roles of PPAR β/δ Regulation in Satellite Cells during Muscle Regeneration

After an injury, satellite cells, as the main adult muscle stem cells, get activated and provide an indispensable role during muscle regeneration [153,154]. The satellite cells and their progeny expand as myogenic precursor cells, where most commit towards terminal differentiation and fuse with existing myofibres, so as to regenerate and restore functional myofibers [154]. A small percentage of these myogenic precursor cells, which do not commit into terminal differentiation, return to a quiescent state, providing a pool of satellite cells so as to sustain the muscle's capacity for future regeneration [155]. Satellite cells are notoriously difficult to study, because of their low abundance under the basal lamina of skeletal muscle. Currently, knowledge of human satellite cells is limited, and most of the studies of satellite cells are performed using mice models [155].

PPAR β/δ has been shown to be important for the proper maintenance of satellite cells, as well as postnatal muscle myogenesis, and it is better studied among the PPAR proteins, because of its abundant expression in skeletal muscle. The specific ablation of PPAR β/δ in the mouse satellite cells has been reported, with approximately 40% fewer satellite cells than their wild-type littermates [156]. A similar observation was also reported in total PPAR β/δ -knock out mice [157]. Mice with PPAR β/δ -deficient muscle progenitor cells exhibited impaired muscle regeneration after cardiotoxin-induced injury and exhibited reduced growth kinetics and proliferation in primary cultures [156]. Furthermore, these mice developed metabolic syndrome upon aging, similar to the PPAR β/δ knockout mice [43,156,157]. The authors found reduced foxhead box protein (FOXO1) expression in quiescent PPAR β/δ -deficient satellite cells, which impaired the proliferation and differentiation ability of these satellite cells during muscle regeneration, thus suggesting that PPAR β/δ regulates the regenerative capability of skeletal muscle through FOXO1 [156]. In addition, CPT1 β expression was also found to be reduced during quiescence, but the differences were abolished on day 5 of muscle regeneration [156], suggesting a possible PPAR β/δ -regulated metabolic role during quiescence [156].

Recent findings on the role of the lipid and glucose metabolism in stem cell cellular homeostasis have been increasingly postulated to be vital in stem cell maintenance and their proliferative activity [158,159]. Delineation of cellular metabolism in satellite cell fate could potentially offer pharmacological strategies in the treatment of degenerative muscle diseases, such as Duchenne muscular dystrophy (DMD). PPAR β/δ has been suggested as a direct transcriptional regulator of utrophin A, a key member of the dystrophin-associated protein complex [160,161]. The expression of utrophin A, stimulated by the PPAR β/δ agonist, GW501516, in the mdx mouse model of DMD has been shown to improve sarcolemma integrity, protect muscles from contraction-induced damage, and help to alleviate muscle wasting, which ultimately slowed down the disease progression [161]. Therefore, understanding the function of PPAR β/δ , and potentially the two other PPAR members, in skeletal muscle progenitor cells has important implications for muscle regeneration and the treatment of degenerative muscle diseases.

8.2. PPAR-Regulated Paracrine Networks between Muscle and Other Cell Types

Inflammation, specifically the infiltration of macrophages during early phases of muscle regeneration, is a major component for efficient healing and repair. Varga et al. [162] showed that myeloid-specific conditional PPAR γ knockout mice exhibited a pronounced delay in muscle regeneration following a toxin-induced injury, compared with their wild-type counterparts. The injured muscle in these mice displayed a reduced muscle differentiation without differences in macrophage infiltration and phagocytic activity. They determined that the macrophage secretion of growth differentiation factor 3 (GDF3), through a direct PPAR γ regulation, is a potent inducer of myotube formation, demonstrating the role of PPAR γ -dependent paracrine signaling between the infiltrated macrophages and regenerating muscle [162].

Skeletal muscle is known to be highly vascularised, and numerous studies have demonstrated the importance of myogenesis and angiogenesis during skeletal muscle regeneration [163–165]. Recent findings on the PPAR β/δ -modulated paracrine network between the endothelial progenitor

cells and regenerating myofibers, have been reported to promote both myogenesis and capillary angiogenesis [165]. PPAR β/δ activation in endothelial progenitor cells promotes insulin-like growth factor 1 (IGF1) signaling pathway in both the skeletal muscle and endothelial cells, via a direct PPAR β/δ induced transcriptional activation of matrix metalloproteinase 9 (MMP9) [165]. Matrix metalloproteinases are well known for their proteolytic activities in the extracellular matrix and they promote angiogenesis [166]. The increased MMP9 secretion from PPAR β/δ agonist-treated endothelial progenitor cells, promotes the (MMP9)-mediated insulin-like growth factor-binding protein 3 (IGFBP3) proteolysis, and thereby modulates the IGF1 activity [165,167]. The MMP9-dependent increase in IGF1 signaling was further demonstrated via the transplantation of PPAR β/δ -activated endothelial progenitor cells to a hindlimb ischaemic mice model. These mice showed an increase in regenerating the myofiber numbers and an enhanced capillary-to-myocyte ratio. The enhanced muscle regeneration and increased angiogenesis promoted a better muscle architecture with reduced fibrosis, and thereby protected the ischaemic limb from hypoxic damage [165].

Interestingly, recent reports on adiponectin produced by skeletal muscle as a myokine, exert anti-diabetic metabolic effects similar to PPAR activation [168]. The skeletal muscle-derived adiponectin has been demonstrated to regulate the fatty acid metabolism, increase glucose uptake, and induce mitochondrial biogenesis, through human skeletal muscle primary culture, muscle biopsies, and gain/loss function studies in rodent models [169–172]. Adiponectin promotes fatty acid uptake and oxidation through a series of sequential activation, involving AMPK, p38 mitogen-activated protein kinase (MAPK), and PPAR α . In skeletal muscle, the activation of AMPK has been known to inhibit lipid biosynthesis through the phosphorylation of acetyl-CoA carboxylase (ACC) [173,174]. Indeed, adiponectin treatment in mouse myotube inhibited ACC phosphorylation in a time-dependent manner [170]. The PPAR γ agonist, rosiglitazone, has been shown to induce adiponectin production and secretion directly [175], and is directly correlated with the rosiglitazone-mediated improvement in insulin sensitivity [176]. The overexpression of PPAR γ in the mouse skeletal muscle also increased adiponectin expression, which protected these mice from high-fat diet induced insulin resistance [117].

9. Regulation of PPARs during Aging

Both physical exercise and aging are two physiological situations that have marked, but opposite, effects on muscle mass. Aging is a complex and multifactorial process that is characterized by progressive, endogenous, and irreversible alterations in cellular signaling, and it is associated with the slow and concerted decline of physiological functions [177]. Moreover, age is the single most significant risk factor for metabolic disorders, such as obesity, T2DM, and other major debilitating and life-threatening conditions [178]. In humans, aging leads to a loss of muscle mass, though the magnitude of loss varies substantially among individuals [179]. Age-related muscle loss is also accompanied by fiber type transformation, metabolic changes, and ectopic fat accumulation over time [180]. In aged muscles, type II glycolytic fibers, particularly type IIx, are susceptible to both atrophy and fiber type switching [180]. Compared to the percentage of glycolytic fibers, an increased percentage of oxidative fibers has been reported in the elderly [181]. Although type I muscle fiber size is largely unaffected [182,183], lower maximal force generation by type I and type IIa fibers was observed in older men, in comparison to that of the similar fibers in younger men [184].

Evidence for the Involvement of PPARs during Aging

In aged muscles, all three PPAR expression levels are decreased and contribute to carbohydrate-lipid metabolism dysregulation, reduced muscle regeneration, and fiber remodeling [185–187]. In addition, the PGC-1 α expression levels, as well as both the oxidative and glycolytic enzymatic capacity, are compromised in the aged skeletal muscle. The age-related decreases in fat oxidation have been consistently associated with reductions in both the quantity and the oxidative capacity to metabolize fats [188]. Lipid metabolism may be further impaired because of the increased lipid accumulation in aged muscle [189]. The decrease in both myonuclear density and mitochondria

numbers in aged muscle has been associated with PPAR β/δ deficiency [43,186]. In rodents, PPAR β/δ overexpression and pharmacological activation stimulate nuclei accretion through the fusion of pre-existing muscle precursor cells to myofibers [186,190]. PPAR β/δ agonist treatment in aged mice restores the muscle fiber distribution profile and the oxidative capacity of the fast-twitch fibers, similar to those of the young untreated counterparts [186].

PPAR α may play a role in glucose utilization in aged muscle. In PPAR α knockout mice, an age-dependent reduction in glycolysis has been observed in the soleus muscle, which comprises mainly of slow-twitch type I fibers [185]. In addition, decreased muscle glycogen concentrations have been detected in aged PPAR α -deficient mice. This suggests a role for PPAR α in modulating metabolic changes during the normal aging process. Interestingly, the clinical use of fibrates may cause muscle weakness and pain (myopathy), or rhabdomyolysis in rare cases [191]. The exact mechanism of PPAR α activation in diseased and aged skeletal muscle remains unclear. However, the mechanism may be partly mediated by the increased oxidative stress and tissue damage associated with PPAR α -induced activity [192,193].

Aging is associated with progressive declines in both insulin sensitivity and glucose tolerance [194,195]. These effects are partly caused by decreased insulin production by the pancreatic islets and deregulated insulin signaling in muscle [196]. The PPAR γ and GLUT4 expression levels are reduced in the skeletal muscle of aged rodents and humans [187,197,198]. In middle-aged adults with both diabetic and non-diabetic histories, insulin-sensitizing TZD compounds improve insulin sensitivity and glucose tolerance, and increase the likelihood of regression from pre-diabetes to normal glucose regulation [199,200]. In aged rodents, rosiglitazone treatment reverses age-related alterations in plasma triglyceride and glucose levels [201]. Paradoxically, in aged animals, mice that were heterogenous for PPAR γ displayed greater insulin sensitivity than their wild-type counterparts [202]. This increased insulin sensitivity was lost upon TZD treatment or high-fat diet administration [203]. The authors suggest that PPAR γ deficiency partially protects from normal physiological age-induced decreases in insulin sensitivity. In short, the physiological impact and role of diminished PPAR γ expression in insulin resistance during the aging process are not clearly understood.

Although PPAR activation has beneficial effects on various metabolic dysfunctions, its beneficial effects on the aging process are not fully understood. More importantly, given the complexity of aging, there are other factors that contribute to aging that have not been discussed here. However, increasing evidence demonstrates that countermeasures can improve age-related metabolic syndromes and muscle loss, partially through modulating endogenous PPAR expression. In addition to pharmacological PPAR activation, interventions such as exercise have been shown to preserve muscle integrity in both aging humans and rodent models. The molecular changes in both lipid and glucose metabolism, after a single bout of exercise in aged humans, have been reported to increase skeletal muscle insulin action [204]. The loss of muscle mass not only reduces mobility and functional capacities which affect the quality of life, but also increases the risks associated with falls and age-related diseases. Developing treatments for age-related and disease-related muscle loss may improve the active life expectancy of older adults, thus leading to substantial health-care savings and an improved quality of life.

10. Concluding Remarks and Perspectives

Numerous studies have provided compelling evidence for important roles of PPAR in skeletal muscle physiology. The capacity to modulate PPAR activity with appropriate agonists or antagonist, further underscores their potential as therapeutic targets. However, the widespread use of these ligands is plagued by their accompanying side effects. Beside myopathy, fibrates are also known to increase the risk for gallstones formation [205] and renal failure [206]. The safety reputation of TZDs suffered as well when the extended use of rosiglitazone and pioglitazone were associated with an increased risk of heart attack/stroke and bladder cancer [207,208]. Although drugs for PPAR β/δ have not been clinically approved, the selective agonist GW501516 has been sold illegally as an endurance booster by its online supplement name, endurobol. GW501516 has been included in the banned

substance list since 2009 by the World Anti-Doping Agency, and was re-categorized as a ‘hormone and metabolic modulator’ drug in 2012. The clinical development of GW501516 was halted in 2007 after increased incidences of several cancer types were observed in rodents [209]. Recent developments in dual- and pan-PPAR agonists displayed therapeutic benefits for the complex and wide-range metabolic disorders [8]. One example is saroglitazar, a dual PPAR α/γ agonist, currently approved in India for the treatment of T2DM and dyslipidemia. Thus, the pharmacological effort in the development of combined PPARs therapeutic effects, with reduced side effects, will be crucial for next-generation drug candidates for metabolic disorders.

Skeletal muscle has been identified as an endocrine organ that expresses and releases myokines as messengers among different organs, as well as within the muscle itself. There are limited studies on the effect of PPAR on the expression of myokines, and even fewer studies on the reciprocal effect of myokines on PPAR expression and activity. For example, the expression of angiopoietin-like 4 (ANGPTL4) is an exercise-responsive myokine and is regulated by PPARs [210,211]. ANGPTL4 may regulate the lipoprotein lipase-dependent plasma clearance of triglyceride from the skeletal muscle during exercise. Another prominent PPAR-regulated myokine is interleukin-6 (IL6), whose expression can be paradoxically exercised-induced or increased during obesity and T2DM [212]. The exact mechanistic involvement of muscle-derived IL6 in health and disease, however, remains elusive, and almost nothing for the IL6 autocrine feedback regulation on PPAR. It is conceivable that pharmacological compounds that mimic the benefits of exercises will also be helpful for elderly adults, as well as for individuals with poignant mobility impairment [213].

The impact of gut microbiota on the whole-body physiology is beginning to be recognized. The bidirectional signaling between the gut microbiota and the brain has been shown to influence neurotransmission and alter behavioral responses through the changes of microbiota-derived metabolites composition. One of the dominant gut-derived metabolites are the short chains fatty acids, such as acetate and propionate, which have been shown to strongly exhibit anti-lipolysis activity in the adipose tissue [214,215].

The gut microbiota and their metabolites or components can modulate the immune system, based on their translocation into tissues and the circulatory system [216]. In recent years, the gut microbiota has been implicated in altered skeletal muscle fiber type proportions in obese porcine, offering a new perspective on the development of dietary supplements for muscle maintenance and regeneration [217]. However, the biological impact, as well as the cause and effect of this gut-muscle connection, remains to be fully understood.

In conclusion, it is clear that PPARs play an essential role in regulating energy homeostasis in skeletal muscle. It is foreseeable that, with a new development in drug design and a better understanding of PPAR's relationship with myokines, among others, PPARs remain important pharmaceutical targets for the therapeutic strategies in order to combat different facets of metabolic syndrome.

Acknowledgments: This research is supported by the Singapore Ministry of Education under its Singapore Ministry of Education Academic Research Fund Tier 1 (2014-T1-002-138-04) to N.S.T., and W.W.T.P is a recipient of the scholarship from the Interdisciplinary Graduate School, NTU, Singapore. We apologize to all fellow scientists whose works were not cited in this review.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

acetyl-CoA	acetyl-coenzyme A
AF1	activation function 1
AF2	activation function 2
AKT/PKB	protein kinase B
AMPK	AMP-activated protein kinase
ANGPTL4	angiopoietin-like 4
ATGL	adipose triglyceride lipase
BMI	body mass index

CD36	cluster of differentiation 36/SR-B2
CPT1	carnitine palmitoyltransferase I
CRY1	Cryptochrome 1
DBD	DNA binding domain
DGAT1	diacylglycerol acyltransferase 1
ER	endoplasmic reticulum
FABP3	fatty acid binding protein 3
FATP	fatty acid transport protein
FATPc	cytoplasmic FABP
FATPpm	plasma membrane FABP
GLUT4	glucose transporter 4
HSL	hormone-sensitive lipase
IGFBP3	insulin-like growth factor-binding protein 3
IL6	interleukin-6
IMCL	intramyocellular lipid
IRS1	insulin receptor substrate 1
LBD	ligand binding domain
LD	lipid droplets
LPL	lipoprotein lipase
MAG	monoacylglycerol
MAPK	mitogen-activated protein kinase
MGL	monoacylglycerol lipase
NRF	nuclear respiratory factor
PDC	pyruvate dehydrogenase complex
PDK	pyruvate dehydrogenase kinase
PGC-1	PPAR γ -coactivator 1
PI3K	phosphatidylinositol-3 kinase
PKC	protein kinase C
PLIN	perilipin
PP2A	protein phosphatase 2A
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator response element
RXR	retinoid X receptors
SCD	stearoyl-CoA desaturase
T2DM	type 2 diabetes mellitus
TA	tibialis anterior
TCA	tricarboxylic acid
TFAM	mitochondrial transcription factor A
TFB1M	mitochondrial transcription factors B1
TFB2M	mitochondrial transcription factors B2
TZD	Thiazolidinediones

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