IntechOpen

Homeostasis

An Integrated Vision

Edited by Fernanda Lasakosvitsch and Sergio Dos Anjos Garnes

HOMEOSTASIS - AN INTEGRATED VISION

Edited by **Fernanda Lasakosvitsch** and **Sergio Dos Anjos Garnes**

Homeostasis - An Integrated Vision
http://dx.doi.org/10.5772/intechopen.71740
Edited by Fernanda Lasakosvitsch and Sergio Dos Anjos Garnes

Contributors

Sheikh Tahir Majeed Majeed, Rabiya Majeed, Ghazia Shah, Khurshid Andrabi, Rabiatul Basria S M N Mydin, Simon Imakwu Okekpa, Fernanda Lasakosvitsch Castanho, Masaaki Murakami, Daisuke Kamimura, Takuto Ohki, Yuki Tanaka, Judith Lechner, Gerhard Gstraunthaler, Kei Nagashima, Ken Tokizawa, Shuri Marui, Yuki Uchida, Ronan Padraic Murphy, Robert Wallace, Michael Harrison, Niall M Moyna, Gerardene Meade-Murphy, Bernard Degryse, Sinead Sheridan, Marc-Antoine Custaud, Nastassia Navasiolava, Laura Twomey, Marco Mangone

Notice

First published in London, United Kingdom, 2019 by IntechOpen
eBook (PDF) Published by IntechOpen, 2019
IntechOpen is the global imprint of INTECHOPEN LIMITED, registered in England and Wales, registration number: 11086078, The Shard, 25th floor, 32 London Bridge Street
London, SE19SG – United Kingdom
Printed in Croatia

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

Additional hard and PDF copies can be obtained from orders@intechopen.com

Homeostasis - An Integrated Vision
Edited by Fernanda Lasakosvitsch and Sergio Dos Anjos Garnes

p. cm.

Print ISBN 978-1-78985-077-2

Online ISBN 978-1-78985-078-9

eBook (PDF) ISBN 978-1-83881-606-3

Meet the editors

Fernanda Lasakosvitsch has a degree in Biological Sciences and a PhD in Sciences from the Department of Microbiology, Immunology and Parasitology with emphasis in Molecular Biology at the Universidade Federal de São Paulo. She has a postdoctoral degree in Molecular Biology from the Universidade de São Paulo. Nowadays she is a researcher and scientific producer of the Funzionali nutrology group, developing studies in enteral nutrition, parenteral nutrition and metabolism in critically ill hospitalized patients.

Sergio dos Anjos Garnes graduated in Medicine from the Federal University of Mato Grosso do Sul, post graduate in Clinical Nutrition, specialist in Nutrology expertise in Parenteral and Enteral nutrition. He is the coordinator of the multi-professional therapy group at the German hospital Oswaldo Cruz in São Paulo and medical director of Funzionali nutrology group, Brazil.

Contents

Preface

In the evolutionary process, physiological mechanisms are developed as a response to the demands of the environment and are in accordance with the ecological factors in which the organisms exist. Diseases such as cancer, type 2 diabetes, autoimmunity, allergy, atherosclerosis, psychic disorder and obesity are a reflection of the mismatch between the environment and human evolutionary history. These diseases have at least one characteristic in common: the disturbance of homeostasis.

The earliest records of an organism having the ability to adjust to maintain its stability are from Hippocrates. He believed that diseases were healed by natural powers, that is, within organisms, there would be mechanisms that would tend to adjust functions when deviated from their natural state.

Most physiological processes operate under a narrow range of conditions, which are maintained by specialized homeostatic mechanisms in response to variations in the environment and are adjusted for changes in functional demands and biological priorities. All types of adaptation are based on homeostatic mechanisms. An organism functioning as an open system may seem extremely susceptible to environmental fluctuations and become unstable but in living beings, the concepts of stability and change assume a prominent role that is essential for the maintenance of life. If there was no stability, there would be no evolution since there would also be no guarantee of maintaining the biochemical characteristics essential to life.

Organisms stay alive because of their ability to stabilize their internal organization, preventing disintegration into an increasingly disorganized state. Maintaining "stability" in the midst of an ever-changing environment requires efficient mechanisms to overcome difficulties, so the living being is an organization in which each disruptive influence induces, per se, the increment of a compensatory activity to neutralize the disturbance.

This book aims to provide the reader an up-to-date view of the self-regulatory mechanisms that are activated to achieve homeostasis, the pathways that are altered during the disease process, and how medicine can intervene to restore balance in critical patients.

Fernanda Lasakosvitsch, PhD
Funzionali Serviços Médicos Ltda
São Paulo, Brazil

Universidade Federal de Sao Paulo
São Paulo, Brazil

Sergio Dos Anjos Garnes
Universidade Federal de Sao Paulo
São Paulo, Brazil

Introduction

Chapter 1

Introductory Chapter: Homeostasis

Fernanda Lasakosvitsch

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.81130

1. Introduction

The human body is composed of several systems and organs, consisting of millions of cells that need relatively stable conditions to function and contribute to the survival of the body as a whole. The maintenance of stable conditions for the cells against the variations of the external environment is an essential function of the body and configures the homeostasis.

In 1870, Claude Bernard described the basic principles of physiological regulation, evidencing the body's need to maintain a stable internal environment. This allows life processes to function optimally during periods of environmental fluctuations and disturbances, and to this end, an array of reflexes and regulatory systems that allow the body to thrive and reproduce despite environmental challenges [1]. Later, Cannon [2] coined the term homeostasis to describe the collective activity of bodily systems that monitor and protect vital life parameters against harmful detours. Many of these processes are unnoticed at the conscious level and depend on behavioral, autonomous, endocrine responses among others to prevent the everyday challenges of the internal environment. Cannon defined homeostasis as "a condition that may vary but remains relatively constant." This balance is guaranteed thanks to the physiological processes that act in a coordinated way in the body and that prevent the changes in the environment interfere in its functioning. Factors such as pH, temperature, plasma osmolality, glucose and calcium are critical for the normal functioning of most organisms and therefore are controlled within narrow limits [3].

The importance of these factors is due to its universal biochemical effects. Temperature and pH may alter the spatial conformation of the structure and function of enzymes and functional RNAs; temperature and plasma osmolality are important in the integrity and function of the membranes; glucose is a key currency for moving and storing energy; and calcium plays key roles in muscle physiology and multiple signaling pathways. The variation of these factors constitutes a noise that affects all physiological systems, including the homeostatic systems

themselves. Since the noise is global (affects all other physiological components in other homeostatic systems), cells cannot filter it or develop private channels to avoid it; instead, the options are to send redundant signals, minimize the spatial and temporal proximity between the transmitter and the receiver, or minimize the variation in the noise-inducing factors. Of all these events, minimizing noise is a key factor that drives the evolution of homeostasis [4].

Until the last decades, homeostasis was considered as a reaction to continuous disturbances in vital systems through negative feedback responses. According to Langley [5], homeostasis is a self-regulating negative feedback system that keeps the internal environment constant. Current views on the subject assume that negative feedback systems that are not activated until a disturbance of the regulated variable occurs may be inefficient and fail in its regulatory role. Negative feedback was the first underlying process used to explain how homeostasis works. This was a reactive strategy through which the perturbation of a regulated variable far from its optimal value was detected and, consequently, provoked corrective responses that served to return the variable back to the pre-disturbance levels. For example, a sudden drop in oxygen content in the blood is detected by sensors that are synaptically linked to areas of the brain that control respiratory rate, and respiration and oxygen in the blood are consequently increased. Although negative feedback is certainly an essential component of regulation, based on past experience, organisms learn to prepare early responses to impending disruption, thereby reducing their impact [4].

A fundamental principle of homeostatic regulation is that the answers work in a coordinated way to defend the body parameters critical to the well-being of an animal; that is, that homeostatic regulation is purposeful. Strategies that may appear to be anticipatory use the initial sensory information in an area of the body as the signal of an event that could potentially disrupt a critical regulated variable, activating corrective effectors and preventing the critical variable from being disturbed. For example, when entering a cold environment, skin temperature sensors are activated before there is any change in core temperature (the variable that is presumably homeostatically regulated); and an effect of increased activity of skin thermometers is to trigger a thermopreflection reflex cycle that induces increased metabolic rate or peripheral vasoconstriction that can stabilize central temperature [6].

If the body cannot maintain the homeostasis of the critical variables, normal function is interrupted and a state, or pathological condition, can develop. Diseases are characterized in two general groups according to their origin: those in which the problem arises from an internal insufficiency or failure of some normal physiological process, and those that originate from some external source. The critical patient develops a condition where the loss of organic homeostasis puts him at immediate risk of death. Therapeutic priorities are focused on correcting these imbalances, such as hemodynamic stabilization, restoration and maintenance of blood volume, stabilization of ventilation conditions and correction of other abnormalities such as electrolyte imbalance, metabolic changes and pain control. Thus, medicine can intervene to restore balance through the activation of self-regulating mechanisms, restoration of the pathways that are altered during the disease process and, finally, to achieve homeostasis in critical patients.

Author details

Fernanda Lasakosvitsch[1,2]*

*Address all correspondence to: flcastanho@gmail.com

1 Funzionali Serviços Médicos Ltda, São Paulo, SP, Brazil

2 Universidade Federal de Sao Paulo, Brazil

References

[1] Bernard C, Noble D. Experimental Physiology-Paton Lecture the first systems biologist, and the future of physiology. Experimental Physiology [Internet]. 2007;**93**:16-26. Available from: https://pdfs.semanticscholar.org/9049/44f321fef4a0996b00c36a1ce643f7ddce96.pdf [cited 2018 Aug 21]

[2] Cannon WB. Organization for physiological homeostasis. Physiological Reviews. 1929;**9**(3):399-431

[3] Carlson AJ. The Wisdom of the Body. Walter B. Cannon. American Journal of Sociology [Internet]. 1933 Jan;**38**(4):651-651. Available from: https://www.journals.uchicago.edu/doi/10.1086/216202 [cited 2018 Aug 21]

[4] Ramsay DS, Woods SC. Clarifying the roles of homeostasis and allostasis in physiological regulation. Physiological Reviews [Internet]. 2014 Apr;**121**(2):225-247. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24730599 [cited 2018 Aug 21]

[5] Langley LL. Homeostasis: Origins of the Concept. 1st ed. In: Langley LL, editor. Michigan: Dowden, Hutchinson & Ross; 1973. 362 p

[6] Morrison SF, Nakamura K. Central neural pathways for thermoregulation. Frontiers in Bioscience [Internet]. 2011;**16**:74-104. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3051412&tool=pmcentrez&rendertype=abstract

Section 2

Physiological Mechanisms of Homeostasis

Chapter 2

Circadian Body Temperature Rhythm and the Interaction with Energy State

Kei Nagashima, Ken Tokizawa, Shuri Marui and Yuki Uchida

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76229

Abstract

We have revealed that circadian body temperature (T_b) rhythm is significantly influenced by fasting/fasting-related hormones. The effect of circadian mechanism and fasting/fasting-related hormones on thermoregulation was examined. Fasting decreases T_b during the light phase in rodents. For the regulation, the suprachiasmatic nucleus (SCN) and clock genes, such as *Cry* and *Clock*, are necessary. In addition, ghrelin and several hypothalamic nuclei, that is, the medial preoptic area, paraventricular nucleus (PVN), and arcuate nucleus (ARC), play a key role in the T_b rhythm. During the light phase, fasting and ghrelin affect the hypothalamic areas. The activity of the SCN increases and that of the ARC decreases. The SCN sends inhibitory signals to the PVN, which may result in a lower heat production in the interscapular brown adipose tissue (iBAT) and T_b. By contrast, during the dark phase, the activity of the SCN decreases and that of the ARC increases. The inhibitory signal from the SCN is less, and the PVN is activated. Heat production of the iBAT increases and T_b is maintained. There are functional and anatomical connections between the circadian and thermoregulation systems. The circadian system modulates thermoregulatory response to hypothermia and/or cold depending on time and feeding condition.

Keywords: fasting, ghrerin, leptin, brown adipose tissue, paraventricular nucleus, arcuate nucleus, suprachiasmatic nucleus

1. Introduction

The temperature of the cell(s), tissues, organs, and body is an important factor that determines the biological functions and survival of organisms, from prokaryotes to vertebrates, although the preferred temperature varies. Homeothermic animals can constantly regulate body temperature (T_b). Thermoregulation is the balance between heat loss and heat

production in the body. The temperature regulation is different among species, and the range of temperature regulation is fairly narrow compared to the larger temperature ranges in the living environment [1].

Homeothermic animals show a circadian T_b rhythm, although the goal of thermoregulation is to maintain a constant T_b. The T_b is higher in the active phase and lower in the inactive phase in both diurnal and nocturnal animals. The T_b rhythm is also observed under conditions wherein the influence of physical activity (i.e., heat production) is minimized. For example, circadian T_b rhythm is observed even in individuals forced to be on complete bed rest [2]. Results suggested that the circadian T_b rhythm can also be regulated.

Despite the usual small amplitude of the circadian T_b rhythm (e.g., less than 1°C in human beings [3]), the rhythm is still important for preserving energy during the inactive phase. The maintenance of higher T_b is energy-costly because more energy from the total daily intake is used for heat production [4]. Therefore, the circadian T_b rhythm may be important in saving energy in homeothermic animals when energy is not needed.

Environmental temperature significantly affects the thermoregulation system. We assessed the circadian T_b change in rats that were placed in an ambient temperature of 18, 25, or 32°C (unpublished data). Laboratory rats are usually housed at 25°C. However, such cold or heat stress did not alter the circadian T_b rhythm. Results suggested that the T_b rhythm is not simply a result of circadian change in heat loss or production in the body. Rather, it is a result of the coordinated thermoregulatory processes that maintain a specific T_b at a given time of the day. Moreover, the T_b rhythm is probably generated by the association between the circadian and thermoregulation system. However, the mechanism is not yet known.

As mentioned previously, the T_b rhythm remains unchanged even when environmental temperature is altered. However, our previous studies have revealed that the T_b rhythm is remarkably influenced by fasting/fasting-related hormones. It is hypothesized that a lack of energy affects thermoregulation and the T_b rhythm. However, several previous studies do not support this hypothesis. In this study, a review of the literature on the mechanism and physiological effects of the circadian T_b rhythm was carried out.

2. Association between fasting and circadian core body temperature rhythm

Fasting is a strong stimulus that changes the amplitude of the circadian T_b rhythm in both mammals and birds [5–8]. In a previous study by Tokizawa et al. [9], T_b was obtained with a thermometer placed in the abdominal cavity of mice, and it was continuously and noninvasively monitored with a telemetry. The most significant reduction was during the light (inactive) phase, whereas the reduction in the dark (active) phase was not remarkable (**Figure 1A**). In addition, no difference was observed in the spontaneous activity during both phases (**Figure 1B**). Results suggested that less heat production due to a decreased physical activity was not associated with the mechanism involved in the reduction of T_b during fasting. Moreover, a remarkable change in T_b was observed at a specific time, which may indicate an association with the circadian rhythm, although the phase shift of the rhythm was not observed.

http://dx.doi.org/10.5772/intechopen.76229

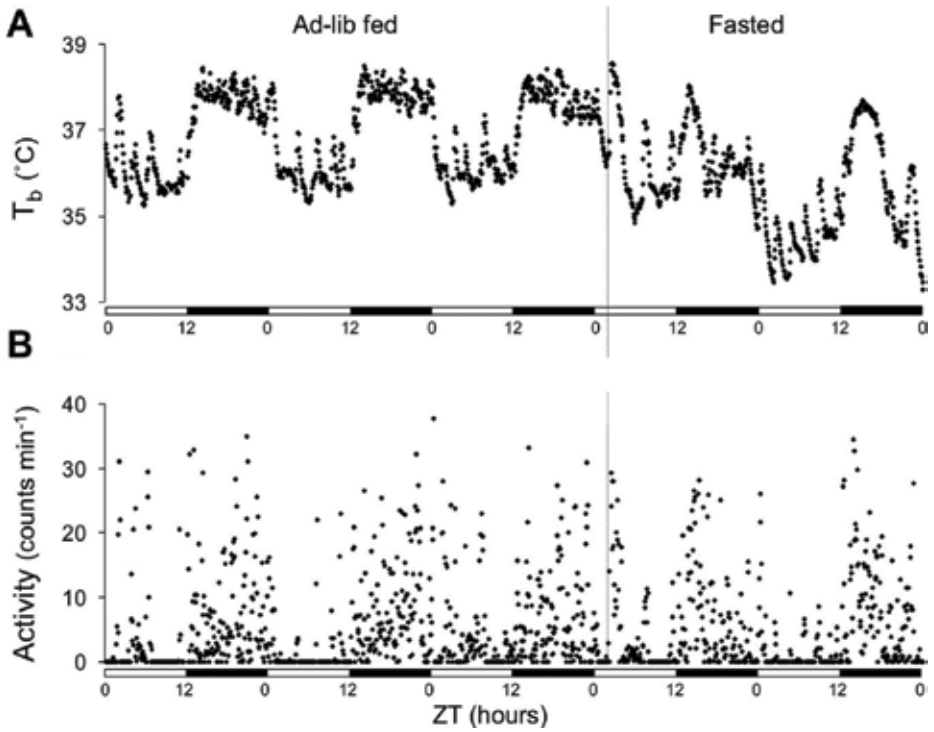

Figure 1. Circadian changes in T_b and spontaneous activity during *ad lib* feeding and fasting in settings with normal temperature (27°C) in mice. The x-axis indicates ZT. The lighting condition is shown at the bottom with the open bar denoting the light phase and the closed bar the dark phase. Data were plotted in 5-min bins. The vertical dashed line shows the time when the 48-h fasting period was started. The modified figure was obtained from the manuscript by Tokizawa et al. [9].

Compared to that during a normal T_b rhythm, the significant decrease in T_b during the light phase (i.e., from 36.0°C during feeding to 34.1°C during fasting) may lessen heat transfer from the body to the environment and preserve energy. In contrast, the maintenance of T_b during the dark phase may be essential in the maintenance of physical activity. On the basis of previous studies, the following questions might be raised: (1) Is the reduction in T_b during the light phase a regulated phenomenon by the thermoregulation system? (2) Is the circadian system involved in the phenomenon? and (3) Which factor that is associated with fasting causes the decrease in T_b, and how?

3. Physiological mechanism involved in the decrease in body temperature during fasting

T_b in homeothermic animals is controlled by heat production and heat loss mechanisms. However, whether such control is maintained and generates the circadian T_b rhythm during fasting is still not verified. Fasting is a strong stimulus that decreases metabolic heat production in rodents and pigeons [5, 6, 10–15]. In addition, fasting decreases thermal conductance

(i.e., opposite of thermal resistance) from the body core to the environment (heat loss [16–18]). If these physiological responses occurred in the same manner during the day of fasting, the reduction of T_b, specifically during the light phase, would not be observed. Therefore, the thermoregulatory responses must be different during the two phases.

Nagashima et al. [19] assessed the T_b, counts of locomotor activity, and oxygen consumption rate ($\dot{V}O_2$) in rats that were made to fast for 3 days. **Figure 2** illustrates the data before fasting and on the last day of fasting and recovery day. T_b and counts of activity were obtained as the

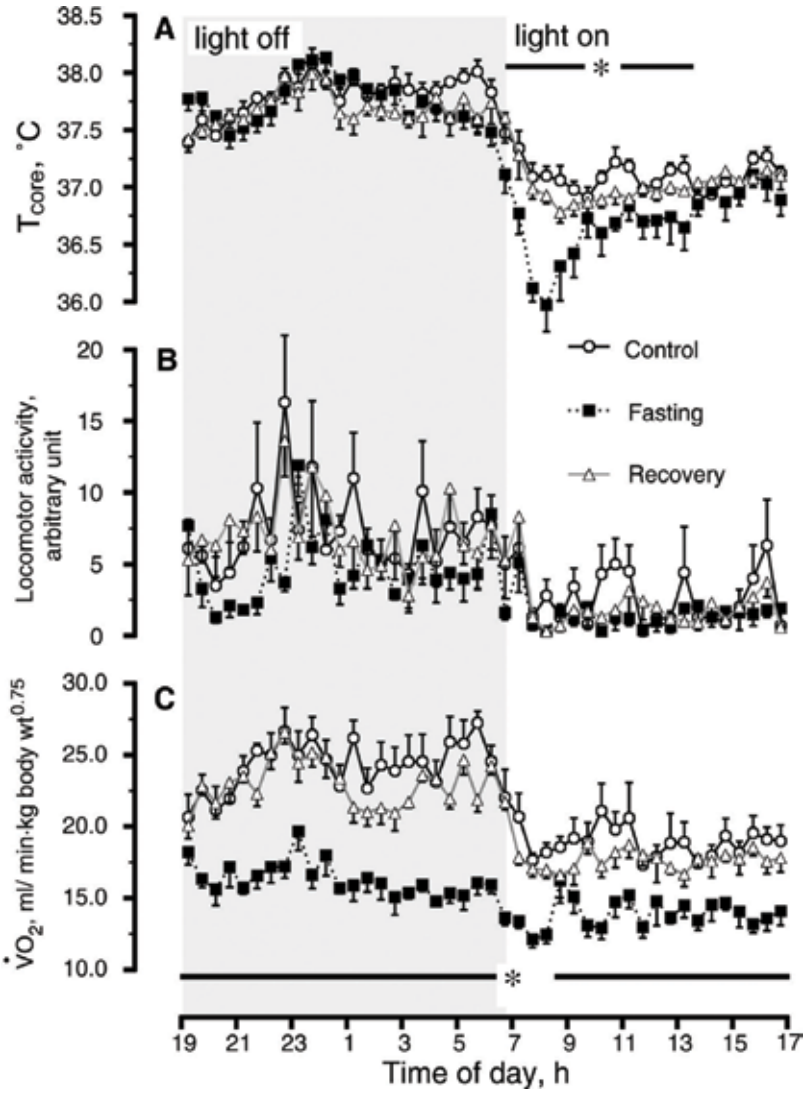

Figure 2. Body core temperature (*A*, T_{core}), counts of locomotor activity (*B*), and oxygen consumption rate (*C*, $\dot{V}o_2$) during feeding, day 3 of fasting, and day 4 of recovery. Stippled area, dark phase (1900–0700). Each point has an average of 30 min. The values are the means ± SE in six rats. *Significant difference from controlled feeding, $P < 0.05$. The figure was obtained from the manuscript by Nagashima et al. [19].

http://dx.doi.org/10.5772/intechopen.76229

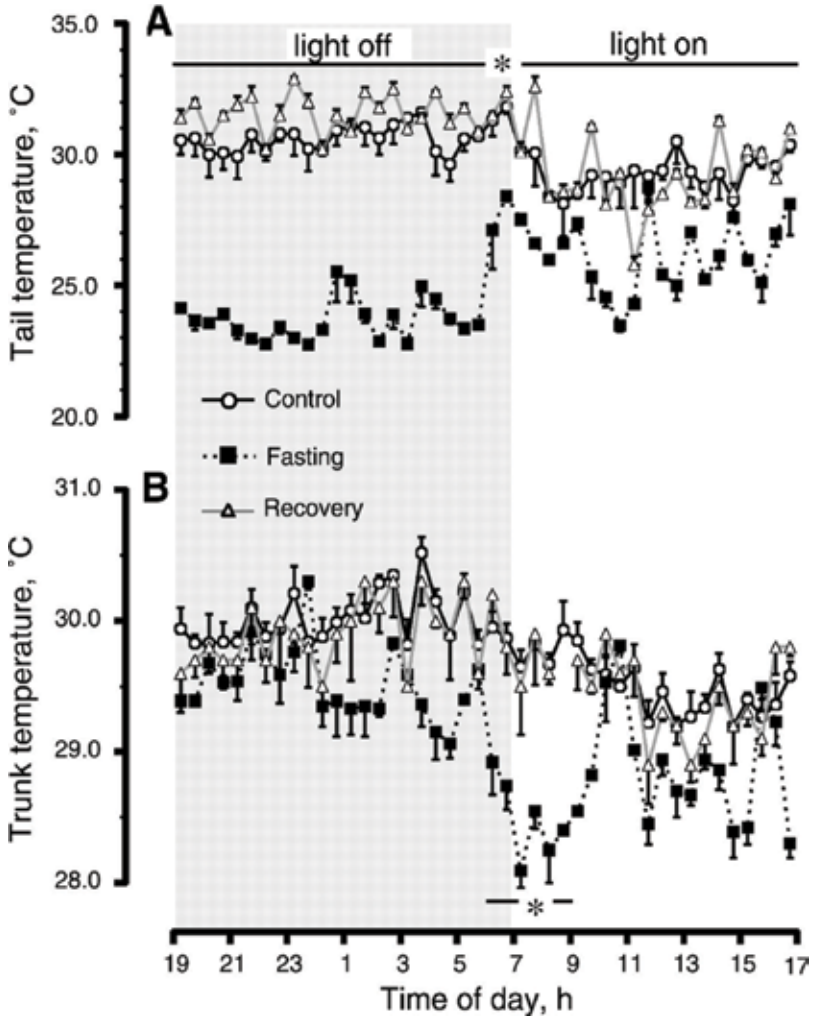

Figure 3. Surface temperatures of the tail (*A*) and trunk (*B*) determined by thermography in a controlled feeding condition, on day 3 of fasting, and day 4 of recovery. T_{tail} is the average of surface temperatures at one-third of the length of the tail from the root and the tip. T_{trunk} is the average of surface temperatures of the head and middle parts of the upper and lower back. Each point has an average of 30 min. The values are the means ± SE in five rats. *Significant differences from controlled feeding conditions, $P < 0.05$. The figure was obtained from the manuscript by Nagashima et al. [19].

study by Tokizawa et al. [9], and $\dot{V}O_2$ was assessed by indirect calorimetry. $\dot{V}O_2$ decreased during fasting in both dark and light phases. Although the rhythms of T_b and $\dot{V}O_2$ were significantly associated during feeding, the amplitude of the $\dot{V}O_2$ rhythm decreased and that of T_b increased during the fasting. The result suggested that T_b was maintained by the suppression of heat loss during fasting and the dark. The estimated thermal conductance during the dark phase of the fasting period decreased from that in the fed condition (1.10 and 1.65 $ml \cdot min^{-1} \bullet kg\ body\ wt^{-0.75} \cdot °C^{-1}$, respectively), which supported the hypothesis. On the contrary, such a change was not observed during the light phase.

Animals use several mechanisms to change the efficiency of heat loss. Among the mechanisms, the tail is a crucial site for the regulation of heat loss in rats and mice, with its physiological and anatomic characteristics, that is, a high density of arteriovenous anastomosis [6], the absence of fur, and a remarkable surface-to-volume ratio. Young and Dawson [20] reported that rats could dissipate 25% of basal heat production by changing the blood flow in the tail.

To assess the contribution of the tail in thermoregulation, Nagashima et al. [19] estimated the tail surface temperature by thermography (**Figure 3**). Tail temperature during the fasting day was lower than that during the fed control day. However, body trunk temperature during the fasting was lower than that during the fed control only around the light-onset period. Interestingly, different from the fed control, tail temperature in the fasting condition increased after light onset and remained at a higher level during the light phase. The results strongly suggested an attenuation of tail blood flow in the dark phase during the fasting condition and were blunted at the beginning of the light phase. Thus, the tail may help in determining the process of heat loss from the body in the fasting condition, which was the factor regulating the T_b rhythm.

4. Involvement of suprachiasmatic nucleus in T_b rhythm during fasting

The suprachiasmatic nucleus (SCN) in the hypothalamus is thought to be the master clock of the circadian rhythm. In addition, electrical or chemical lesions of the SCN destroy the T_b rhythm in rodents [21–26]. However, these findings may not indicate that the circadian clock regulates the T_b rhythm. Behavioral and/or physiological responses, such as locomotor activity, eating, and the secretion of several hormones, are also inhibited due to the SCN lesion, which may have direct influences on heat production and T_b [17, 27–29]. Simply put, SCN lesion inhibits such behavioral and/or physiological responses [3, 21, 24, 30], and the T_b rhythm is subsequently destroyed.

Liu et al. [23] assessed the circadian T_b rhythm within 4 days of fasting in rats, of which SCN was electrically lesioned. The rats had arrhythmia of T_b and spontaneous activity, and no difference was observed during the light and dark phases and the non-fasting and fasting periods. Although the experiment did not answer the question of whether the circadian clock directly regulates the T_b rhythm, the result suggested that SCN may be important for the changes in thermoregulation and T_b due to fasting.

5. Importance of molecular circadian mechanism on T_b rhythm during fasting

Several genes in the central and peripheral tissues have expression rhythms with a periodicity of ~24 h. Among these genes, those with autoregulatory transcription-translation loops with a periodicity of ~24 h may be responsible for the core molecular mechanisms of the circadian clock (i.e., clock genes). These genes are observed in both the central and the peripheral tissues [31–33].

Nagashima et al. [34] assessed the circadian T_b rhythm in mice that lack the cryptochrome 1 and 2 genes (*Cry*1 and *Cry*2), two of the core clock genes [35–38]. The mice loose the transcription-translation rhythms of the *Cry*1 and *Cry*2 and other clock genes and periodicity in a wheel-running behavior [38], as well as the electrophysiological activity of the SCN cells under constant dark conditions [39]. However, the behavior is suppressed under the light condition, and the daily rhythm is observed under both light and dark conditions. Therefore, despite the lack of the internal rhythm, lighting rhythm could induce T_b rhythm.

Table 1 summarizes the cosinor rhythm analysis [34] of T_b, spontaneous activity, and $\dot{V}O_2$ in normal wild-type mice that lack *Cry*1/*Cry*2 ($Cry1^{-/-}/Cry2^{-/-}$). The mice were placed under three different conditions: (1) constant darkness (DD) with ad lib feeding, (2) 12–12-h light-dark (LD; lights on at 1900) cycle with ad lib feeding, and (3) LD with food restriction. The food restriction protocol aimed to enhance the eating rhythm. Ordinary chow was given at 1900, which is the end of the light period, at 70–80% of the normal daily intake. Both $Cry1^{-/-}/Cry2^{-/-}$ and wild-type mice finished eating 10–12 h after the appearance of chow in the food-restriction regimen.

In the wild-type mice, no significant difference was observed between the mean, amplitude, and peak phase of the T_b rhythm under the LD condition with ad lib feeding and DD condition. Differences were found in the T_b and $\dot{V}O_2$ rhythms between ad lib feeding and food-restriction days (i.e., greater amplitude of the rhythms during the food-restriction day). The activity rhythm did not change throughout the three conditions. In $Cry1^{-/-}/Cry2^{-/-}$ mice, T_b, $\dot{V}O_2$, and spontaneous activity were arrhythmic under the DD condition. However, T_b, $\dot{V}O_2$, and spontaneous activity became rhythmic in the same peak phases during the LD conditions. As for the T_b and $\dot{V}O_2$ rhythms in the LD condition, the daily means were lower, and the amplitudes of the rhythms were higher in the food-restriction condition than those under the ad lib feeding condition. The study showed that the circadian T_b rhythm is observed even in mice that lack the internal circadian mechanism, when an external lighting and feeding stimuli that alter heat production are observed. The result showed that the heat production rhythm may be a key component for the T_b rhythm.

	T_{core}, °C			$\dot{V}O_2$, ml · min^{-1} · kg body wt$^{-0.75}$			Activity, Au		
	Fed	Fast	Rec	Fed	Fast	Rec	Fed	Fast	Rec
Mesor	37.5 ± 0.1	37.3 ± 0.1*	37.4 ± 0.1	21.63 ± 0.84	15.02 ± 0.55*	20.11 ± 0.78‡	5.0 ± 1.0	3.2 ± 0.3	5.0 ± 0.9
Amplitude	0.5 ± 0.1	0.7 ± 0.1*	0.4 ± 0.1*,‡	3.74 ± 0.44	1.57 ± 0.27*	3.06 ± 0.38*,‡	3.4 ± 0.6	2.6 ± 0.3	3.5 ± 0.5‡
Acrophase	18.2 ± 0.3	16.4 ± 0.3*	17.5 ± 0.3*,‡	18.2 ± 0.2	16.4 ± 0.3*	17.6 ± 0.4*,‡	17.7 ± 0.4	17.9 ± 0.4	17.3 ± 0.6
r	0.76–0.91†	0.80–0.85†	0.56–0.91†	0.51–0.85†	0.36–0.61†	0.45–0.74†	0.38–0.67†	0.39–0.63†	0.43–0.67†

Values are means ± SE. $\dot{V}O2$, oxygen consumption rate; Au, arbitrary unit; T_{core}, core temperature; Fed, *day* 4 of fed control period; Fast, *day* 3 of fasting period; Rec, *day* 4 of recovery from fasting. Acrophase is shown in zeitgeber time (ZT, ZT 0 = 0700).

*Significantly different from control, $P < 0.05$.

†Significant regression coefficient (r) for fitted cosine curve for daily change of variable, $P < 0.05$.

‡Significantly different from fasting period, $P < 0.05$.

Table 1. Analysis of cosinor rhythmometry for daily changes in core temperature, Vo_2, and locomotor activity.

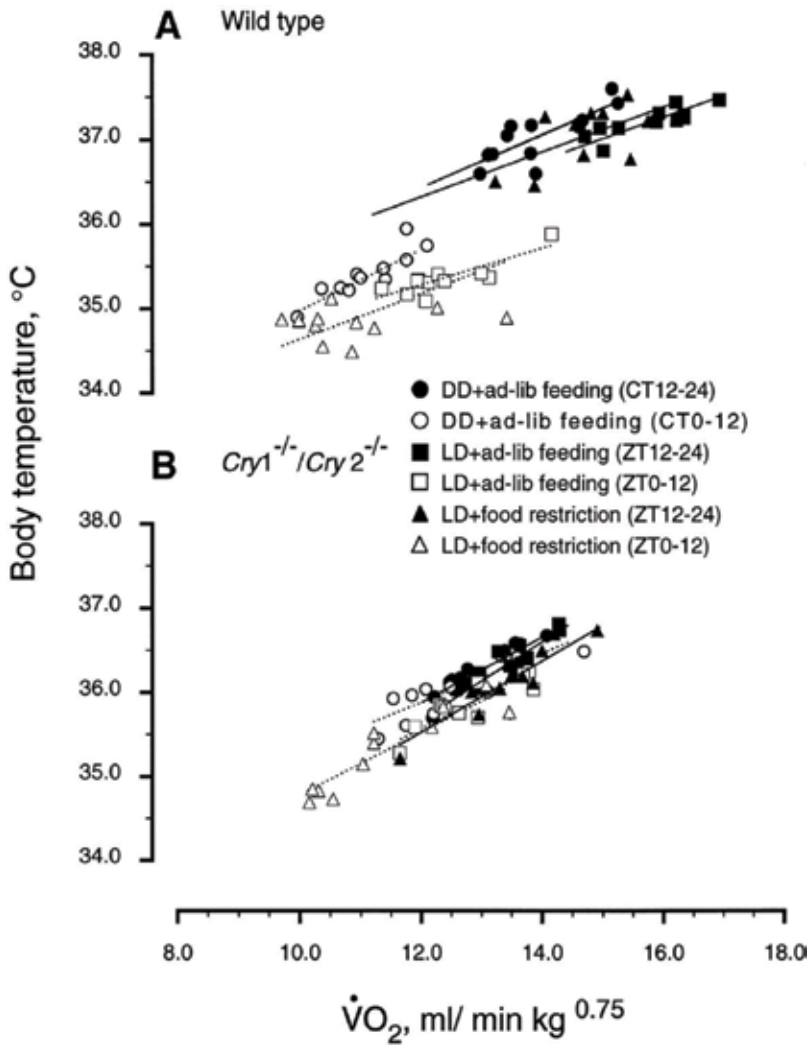

Figure 4. Relationship between metabolic heat production ($\dot{V}O_2$) and T_b in wild-type mice (*A*) and *Cry*1$^{-/-}$/*Cry*2$^{-/-}$ (*B*) based on three trials. The values are the means (±SE) in five mice for 30 min, and data corresponding to each hour are used for this illustration. Regression lines were applied for the averaged values in each group. The figure was obtained from the manuscript by Nagashima et al. [34].

Figure 4 shows the results of the regression analysis of the $\dot{V}O_2$ and T_b of each group. If heat production was the sole determinant of circadian T_b rhythm, these regressions must be identical regardless of trial, circadian phase, and group. In addition, model equations fitted to all the data in the three trials within the same phase and the group was established. Model equations were also created for all possible associations among the three trials. The analysis showed that the data could be classified into four groups: those at circadian time (CT; a standard of time based on the free-running period of a rhythm). The onset of activity of diurnal organisms defines circadian time 0, CT 0. The onset of activity of nocturnal organisms defines circadian time 12, CT 12, or zeitgeber time (ZT; under standard light–dark cycles, the time of lights on defines zeitgeber time 0 and the time of lights off defines zeitgeber time 12), 12–24

and CT or ZT 0–12 in *Cry*1$^{-/-}$/*Cry*2$^{-/-}$ mice and wild-type mice, respectively. Moreover, the model indicated that the two regressions in the wild-type mice were different (i.e., for the active and inactive phases), and those in the *Cry*1$^{-/-}$/*Cry*2$^{-/-}$ mice were similar. The results suggested that the circadian phase can be a significant factor in determining the T_b rhythm in wild-type mice but not in the *Cry*1$^{-/-}$/*Cry*2$^{-/-}$ mice. Thus, T_b was kept higher in the active phase than in the inactive phase regardless of the $\dot{V}O$ level in wild-type mice. Moreover, the regression slopes of $\dot{V}O$ and T_b in both phases were smaller than those of the *Cry*1$^{-/-}$/*Cry*2$^{-/-}$ mice (wild-type: 0.24 and 0.25 in the active and inactive phases, respectively; *Cry*1$^{-/-}$/*Cry*2$^{-/-}$: 0.44). Results showed that the wild-type mice can maintain their T_b within a narrower range than the *Cry*1$^{-/-}$/*Cry*2$^{-/-}$ mice over the same variation of $\dot{V}O$ in each phase. Thus, the circadian T_b rhythm in wild-type animals is not a simple byproduct of the heat production rhythm but a phenomenon regulated by the circadian system.

The mammalian sirtuins (SIRT) modulate the circadian epigenome and provide specificity in transcriptional control [40–43]. It was reported that the circadian clock regulates mitochondrial oxygen consumption rate in the oxidoreductase factor nicotinamide adenine dinucleotide (NAD^+)/SIRT3-dependent manner. In addition, NAD^+-dependent enzymes are important in fasting and oxidative metabolism. Peek et al. [40] evaluated NAD^+ biosynthesis, lipid and glucose oxidation, and acetylation of mitochondrial proteins in normal and circadian (Bmal1)-mutant mice. They reported that lipid oxidation and mitochondrial protein acetylation exhibited circadian oscillations that corresponded with the clock-driven NAD^+ cycle in the liver; however, rhythmic NAD^+ and oxidative cycles were self-sustained in fasted mice. These results suggest a strong interaction between circadian and metabolic rhythms and its destruction during fasting but do not explain for the change in circadian T_b rhythm during fasting.

6. Role of the circadian system and associated brain areas in controlling the circadian T_b rhythm during fasting

Tokizawa et al. [9] tested the hypothesis that thermoregulation is modulated by the circadian system (including the SCN and clock genes), depending on the time of day and feeding condition. Moreover, the physiological and neural responses of the mice during exposure to cold (20°C) under ad lib feeding and during 48-h fasting conditions and the dark and light phases were compared. The differences in the responses between wild-type and *Clock*-mutant mice were also examined. *Clock* is also a gene that organizes the core loop of molecular circadian oscillation. The mutation of *Clock* causes the disappearance of oscillation [44]. However, mutant mice show a T_b rhythm under light-dark conditions because of the masking effect of light.

During ad lib feeding at a low temperature setting (20°C), the T_b of the wild-type mice was similar to that of the wild-type mice during both the dark and light phases at normal temperature settings (27°C) (**Figure 5A** and **B**). However, $\dot{V}O_2$ was higher during the light phase at settings with a temperature of 27°C (ZT2.5–3.5; **Figure 5E**). During fasting at low temperature settings, the reduction in T_b increased during both the light and dark phases (ZT1–4 and 14.5–16, respectively; **Figure 5A** and **B**). However, T_b increased during the light phase. $\dot{V}O_2$ increased in settings with a temperature of 27°C at ZT13.5–14.5 during the dark phase (**Figure 5F**) but remained unchanged during the light phase (**Figure 5E**).

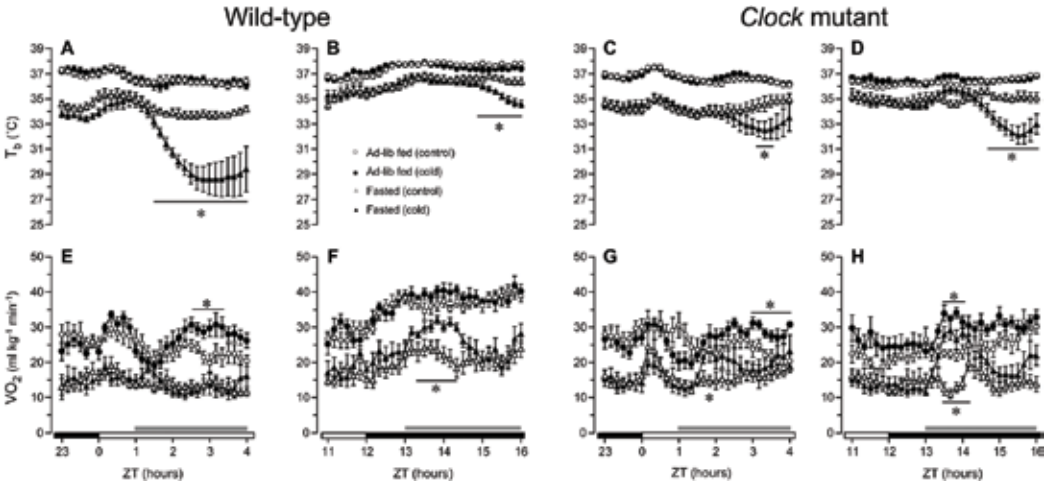

Figure 5. T_b and $\dot{V}O_2$ at low-temperature settings (27 or 20°C) with *ad lib* feeding or fasting in the wild-type and *Clock*-mutant mice (*A–H*). The values given are the means ± SEM ($n = 8$). $^{*}P < 0.05$, 20°C (low-temperature setting) versus 27°C (control setting) in each feeding condition. $^{\#}P < 0.05$, versus the dark phase in wild-type mice and both phases in *Clock*-mutant mice; $^{\S}P < 0.05$, versus dark phase in wild-type mice and light phase in *Clock*-mutant mice. The modified figure was obtained from the manuscript by Tokizawa et al. [9].

During ad lib feeding at low temperature settings, T_b in *Clock*-mutant mice was also maintained at the 27°C level (**Figure 5C** and **D**). $\dot{V}O_2$ increased above the 27°C level during both the dark and light phases (ZT3–4 and 13.5–14; **Figure 5G** and **H**), and no difference was observed between the two phases. During fasting at low temperature conditions, T_b decreased below the 27°C level at ZT3–3.5 during the light phase and ZT14.5–16 during the dark phase (**Figure 5C** and **D**), and no significant difference was observed between the phases. $\dot{V}O_2$ was higher than the 27°C level at ZT2 and 13.5–14 (**Figure 5G** and **H**). This increase in $\dot{V}O_2$ was higher during the dark phase than during the light phase.

The study indicated that the physiological response of mice to low temperature conditions is different during ad lib feeding and fasting in wild-type animals and during the dark and light phases. However, such differences were not observed or significantly decreased in *Clock*-mutant mice. The thermoregulatory mechanism of heat production is attenuated or inhibited during fasting and light phases. For such response, the circadian system is important. To support this result, the expression of uncoupling protein 1 (UCP1) mRNA [45–47] in the interscapular brown adipose tissue (iBAT, one of the effector organs for thermoregulatory heat production) was suppressed during the light phase and fasting in wild-type animals. However, cold exposure increased the expression during both dark and light phases in *Clock*-mutant mice.

7. Histological evidence on functional and anatomical connection between the circadian and thermoregulation systems

On the basis of the physiological findings, the association between the circadian and thermoregulation systems may be significant during fasting. The center of thermoregulation is thought to be in the hypothalamus [48, 49]. However, whether functional and anatomical

neural connections between the SCN exist (the center of the circadian system) and the hypothalamic subregion is involved in the thermoregulation remains unclear.

Liu et al. [23] assessed the cFos (i.e., early gene expression protein) expression in the SCN of rats. cFos can be a marker of neuronal activation in the brain [50]. The number of cFos immunoreactive (cFos-IR) cells changes daily, which is higher during the light phase and smaller during the dark phase. A 4-day fasting did not change the phase difference in the number of cFos-IR cells. However, the number increased during the dark phase and decreased during the light phase. Whether such change in the SCN causes altered T_b rhythm during fasting remains unknown. However, results showed that the SCN receives some information that is associated with fasting and alters T_b rhythm and/or the thermoregulatory responses.

Tokizawa et al. [9] evaluated cFos expressions in the SCN and other hypothalamic areas in wild-type and *Clock*-mutant mice. The mice were exposed to settings with a temperature of 20°C and/or 48-h fasting. Neural associations were also observed between the SCN and the hypothalamic areas involved in thermoregulation. Data are summarized in **Figure 6**. Fasting increased the number of cFos-IR cells in the SCN in both wild-type and *Clock*-mutant mice. The number was smaller in *Clock*-mutant mice than in wild-type mice. cFos-IR cells also increased during fasting in other hypothalamic areas, such as the medial preoptic nucleus (MPO), dorsomedial hypothalamus (DMH), paraventricular nucleus (PVN), and dorsal subparaventricular zone (dSPZ), in wild-type mice. Differences were observed in the number of cFos-IR cells during the dark and light phases (**Figure 6A**, **B**, **D**, **E**). Small increases were also observed in the DMH, ARC, and PVN in *Clock*-mutant mice. However, no phase differences were observed. Neural outputs from the SCN, including the SPZ, reached these hypothalamic areas [51, 52]. Thus, the activation of the SCN during fasting may be linked with the activation of the hypothalamic areas.

The preoptic area in the hypothalamus is thought to be important in thermoregulation because it has several thermosensitive neurons in the core body and skin temperatures [53]. In addition, stimulatory and inhibitory signals are sent from the area to other brain areas, which regulate the effector organs of thermoregulation, such as vasodilation, shivering, and non-shivering thermogenesis [48, 49]. The sympathetic outflow may originate from the PVN [54–56]. The DMH receives thermal input from the skin and is associated with the control of BAT thermogenesis [57]. Whether all cFos-IR cells are associated with circadian change of T_b and/or thermoregulation is not verified. However, the changes in cFos expression between the phases may, in part, be responsible for the decrease in T_b that was observed during fasting and the light phase.

Fasting increased the cFos expression in the ARC in mice [58, 59]. The ARC is involved in the regulation of food intake and energy expenditure, which responds to peripheral nutritional signals, such as the levels of leptin and insulin [60]. However, no study on the direct association between ARC and thermoregulation was conducted. The ARC had phase differences in cFos expression in wild-type mice (**Figure 5C**), which increased during fasting. The ARC has neural input from the SCN [61–63]. Therefore, the fasting signals received by the ARC increased during the dark phase and were attenuated during the light phase in wild-type mice, and this may be related to the signals from the SCN.

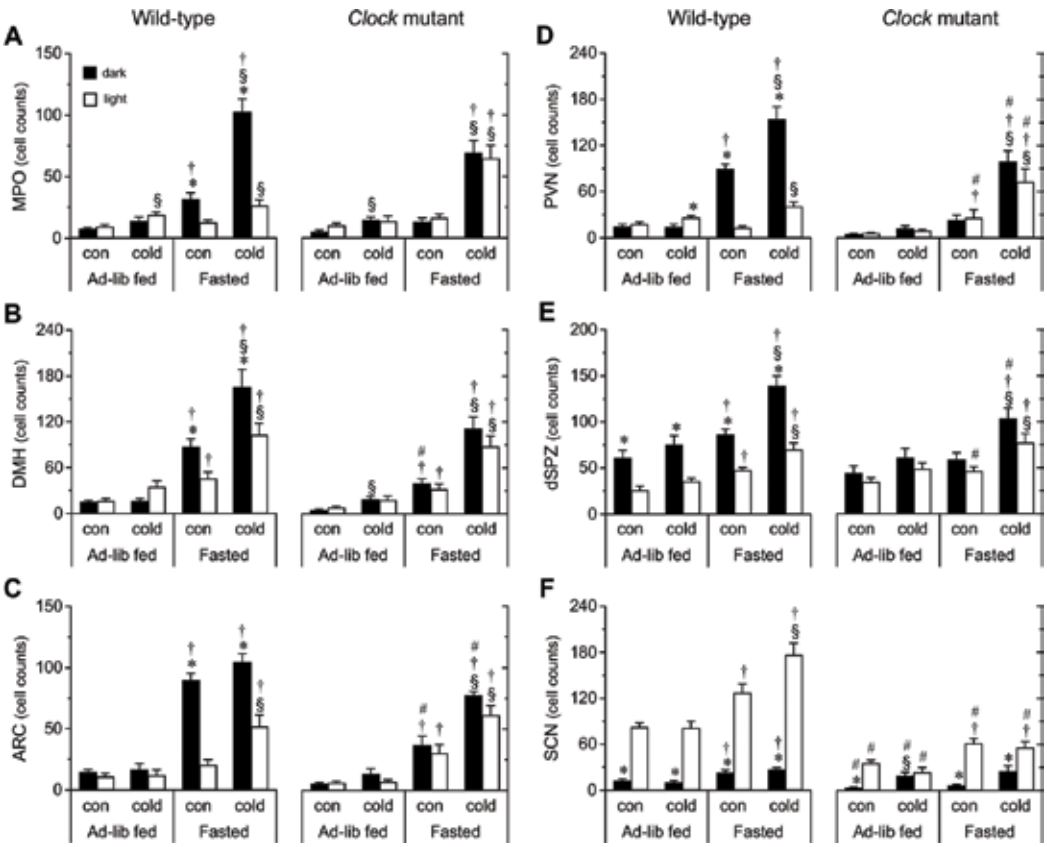

Figure 6. Counts of cFos-IR cells in the MPO (*A*), dorsomedial hypothalamus (*B*, DMH), ARC (*C*), PVN (*D*), dorsal subparaventricular zone (*E*, dSPZ), and SCN (*F*) under various conditions. Values are the means ± SEM ($n = 8$). $^{*}P < 0.05$, dark versus light phase; $^{\S}P < 0.05$, 20°C (low-temperature setting) versus 27°C (control setting); $^{\dagger}P < 0.05$, fasting versus *ad lib* feeding; $^{\ddagger}P < 0.05$, wild-type versus *Clock*-mutant mice. The figure was obtained from the manuscript by Tokizawa et al. [9].

The increase in cFos expression in *Clock*-mutant mice that was attributed to low temperature during fasting was also observed in all the hypothalamic areas besides the SCN. However, no differences were observed between the two phases. In wild-type mice, the number of cFos expression in the SCN increased during the light phase. However, the increases in the MPO and PVN in wild-type mice were lower than those in Clock-mutant mice. Therefore, exposure to cold while fasting increases neural activity in the SCN during the light phase. In addition, normal molecular circadian mechanisms may be necessary for the response. On the basis of the results of cFos expression in the hypothalamic areas, the SCN may send inhibitory signals to the MPO and PVN, which may result in attenuated thermoregulatory responses. Moreover, since the inhibitory signals are stronger in some conditions, such as in fasting, the attenuation of thermoregulation becomes stronger.

On the basis of the speculated neural connection between the SCN and MPO and/or PVN, Tokizawa et al. [9] conducted an experiment, which directly evaluated the associations. A cholera toxin b-subunit was injected (CTb; monosynaptic retrograde neural tracer) to the MPO or PVN during the light phase, and the presence in the SCN was assessed 3 days later. Cold exposure

and fasting were also conducted in the same manner as the previous cFos study. The injection of CTb in the PVN resulted in a widely spread labeling in both the dorsomedial and the ventrolateral parts of the SCN of both wild-type and *Clock*-mutant mice. In both mice, 5–10% of CTb-labeled neurons in the SCN were also cFos-positive at 27°C during the light phase and ad lib feeding. In the cold exposure during fasting, the ratio of the double-labeled neurons of CTb and cFos increased to 25–30% only in the wild-type mice. Moreover, the double-labeled neurons are GABAergic. When CTb was injected to the MPO, the dorsomedial part of the SCN was labeled. However, the ratio of the double-labeled neurons remained at the same level (15–20%) during ad lib feeding and during fasting and cold exposure. These results suggested that the SCN may send inhibitory signals to the PVN and MPO during the light phase. Fasting and/or cold exposure increased the inhibitory signals only in the PVN. Moreover, in the process, a normal molecular circadian mechanism would be necessary. The sympathetic outflow may originate from the PVN [54–56]. Therefore, such inhibitory signals may attenuate metabolic heat production and/or skin vasoconstriction (i.e., cold defense mechanisms), thus decreasing T_b during fasting.

8. Signals attenuating thermoregulatory responses during fasting

In Section 6 of the cFos study, the ARC also seems to play a key role in the circadian change of thermoregulation during fasting, which decreases plasma leptin and increases plasma ghrelin [64]. A reduction of leptin results in hypothermia [65]. Gluck et al. [66] showed that ghrelin induces hypothermia. Decreases in both hormones are signals activating neuropeptide Y (NPY) neurons in the ARC [67], which strongly reduces heat production. The receptors for leptin and ghrelin are found in NPY neurons [68, 69]. These experimental results suggest that leptins and/or ghrelins are the factors that modulate thermoregulatory heat production and decrease T_b during fasting. Moreover, the neurons in the SCN have leptin and ghrelin receptors [68, 70, 71]. In addition, the neural activity of the SCN is modulated in the presence of leptin and ghrelin [72, 73].

Tokizawa et al. [74] reported that the thermoregulatory response to cold was attenuated in *ob*/*ob* mice (genetically deficient of leptin). However, the response was not different between the light and dark phases. On the contrary, ghrelin injection to normal mice inhibited thermoregulatory heat production during cold exposure and reduced T_b. Such a response was observed only during the light phase. Therefore, ghrelin plays a key role in the phase-specific (light phase) modulation of thermoregulation and T_b during fasting.

The administration of ghrelin suppresses sympathetic nerve activity and iBAT temperature in rats [75, 76]. In the study by Tokizawa et al., ghrelin levels after the injection and 48-h fasting did not differ between the two phases. Therefore, a central mechanism that modulates the sensitivity to plasma ghrelin levels must exist, which may affect thermoregulatory responses to cold exposure. Ghrelin induced phase-specific changes in cFos expression in the hypothalamic areas: increased cFos-IR cells in the SCN during the light phase, the ARC during the dark phase, and the PVN during the cold exposure in the dark phase. Ghrelin injection activates NPY neurons in the ARC in both phases, and cFos-IR cell counts are higher in the dark than in the light phase. In the SCN, the ghrelin effect was limited to the light phase, and 25% of the cFos-IR cells were NPY neurons. NPY acts as nonphotic stimuli in the SCN [77]. The activation of the hypothalamic nuclei, that is, the SCN, ARC, and PVN, seems to be involved in the changes in the thermoregulatory metabolic heat production. **Figure 7** shows the summary of the findings.

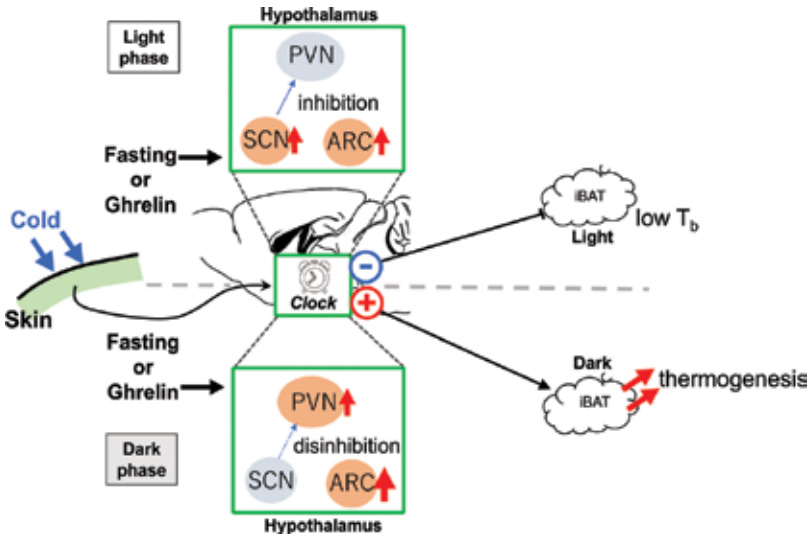

Figure 7. Summary of the histological findings together with physiological studies. During the light phase, fasting and an increase in plasma ghrelin level affect the hypothalamic areas. The activity of the suprachiasmatic nucleus (SCN) increases and that of the arcuate nucleus (ARC) relatively decreases. The SCN sends inhibitory signals to the paraventricular nucleus (PVN), which may result in a lower metabolic heat production of the interscapular brown adipose tissue (iBAT) and a lower body temperature. On the contrary, during the dark phase, the activity of the SCN decreases and that of the ARC relatively increases. The inhibitory signal from the SCN is less, and the PVN is activated. Metabolic heat production of the iBAT increases and body temperature is maintained.

9. Conclusion

This is a review article showing our previous series of studies involved in fasting-induced change in T_b rhythm. Interestingly, the change in T_b rhythm is a regulated phenomenon by molecular mechanisms such as *Cry and Clock* and neural mechanisms such as the SCN, MPO, and ARC in the hypothalamus. These studies are important in considering physiological importance and mechanism of the circadian body temperature and metabolic rhythms.

Acknowledgements

This research was partially supported by the Ministry of Education, Science, Sports, and Culture, Japan; Grant-in-Aid for challenging Exploratory Research, No. 16 K13055; and Ibuka Foundation, Waseda University.

Conflict of interest

There is no conflict of interest in this review.

Author details

Kei Nagashima[1]*, Ken Tokizawa[2], Shuri Marui[1] and Yuki Uchida[3]

*Address all correspondence to: k-nagashima@waseda.jp

1 Body Temperature and Fluid Laboratory, Faculty of Human Sciences, Waseda University, Tokorozawa, Saitama, Japan

2 National Institute of Occupational Safety and Health, Kiyose, Tokyo, Japan

3 Women's Environmental Science Laboratory, Faculty of Human Life and Environment, Department of Health Sciences, Nara Women's University, Nara-City, Nara, Japan

References

[1] Tokizawa K, Yoda T, Uchida Y, et al. Estimation of the core temperature control during ambient temperature changes and the influence of circadian rhythm and metabolic conditions in mice. Journal of Thermal Biology. 2015;**51**:47-54. DOI: 10.1016/j.jtherbio.2015.03.005

[2] Yu X, Chang S, Song D, et al. Body temperature changes during 7 d −6 degrees head-down bed rest. Space Medicine & Medical Engineering (Beijing). 2002;**15**:167-169

[3] Aschoff J. Circadian control of body temperature. Journal of Thermal Biology. 1983;**8**:143-147

[4] Ocobock C. Human energy expenditure, allocation, and interactions in natural temperate, hot, and cold environments. American Journal of Physical Anthropology. 2016;**161**:667-675. DOI: 10.1002/ajpa.23071

[5] Graf R, Krishna S, Heller H. Regulated nocturnal hypothermia induced in pigeons by food deprivation. The American Journal of Physiology. 1989;**256**:R733-738. DOI: 10.1152/ajpregu.1989.256.3.R733

[6] Rashotte M, Basco P, Henderson R. Daily cycles in body temperature, metabolic rate, and substrate utilization in pigeons: Influence of amount and timing of food consumption. Physiology & Behavior. 1995;**57**:731-746

[7] Sakurada S, Shido O, Sugimoto N, et al. Autonomic and behavioural thermoregulation in starved rats. The Journal of Physiology. 2000;**526**(Pt 2):417-424

[8] Yoda T, Crawshaw L, Yoshida K, Su L. Effects of food deprivation on daily changes in body temperature and behavioral thermoregulation in rats. The American Journal of Physiology. 2000;**278**:R134-139. DOI: 110.1152/ajpregu.2000.278.1.R134

[9] Tokizawa K, Uchida Y, Nagashima K. Thermoregulation in the cold changes depending on the time of day and feeding condition: Physiological and anatomical analyses

of involved circadian mechanisms. Neuroscience. 2009;**164**:1377-1386. DOI: 10.1016/j.neuroscience.2009.08.040

[10] Döring H, Schwarzer K, Nuesslein-Hildesheim B, Schmidt I. Leptin selectively increases energy expenditure of food-restricted lean mice. International Journal of Obesity and Related Metabolic Disorders. 1998;**22**:83-88

[11] Munch IC, Markussen NH, Óritsland NA. Resting oxygen consumption in rats during food restriction, starvation and refeeding. Acta Physiologica Scandinavica. 1993;**148**:335-340. DOI: 10.1111/j.1748-1716.1993.tb09564.x

[12] Overton JM, Williams TD, Chambers JB, Rashotte ME. Cardiovascular and metabolic responses to fasting and thermoneutrality are conserved in obese Zucker rats. American Journal of Physiology. Regulatory, Integrative and Comparative Physiology. 2001;**280**:R1007-R1015

[13] Rothwell NJ, Stock MJ. Effect of chronic food restriction on energy balance, thermogenic capacity, and brown-adipose-tissue activity in the rat. Bioscience Reports. 1982;**2**:543-549. DOI: 10.1007/BF01314214

[14] Sakurada S, Shido O, Sugimoto N. Autonomic and behavioural thermoregulation in starved rats. Journal of Physiology. 2000;**526**:417-424

[15] Williams TD, Chambers JB, May OL, et al. Concurrent reductions in blood pressure and metabolic rate during fasting in the unrestrained SHR. American Journal of Physiology. Regulatory, Integrative and Comparative Physiology. 2000;**278**:R255-R262

[16] Markussen NH, Oritsland NA. Metabolic depression and heat balance in starving Wistar rats. Comparative Biochemistry and Physiology. A, Comparative Physiology. 1986;**84**:771-776

[17] Phillips DL, Rashotte ME, Henderson RP. Energetic responses of pigeons during food deprivation and restricted feeding. Physiology & Behavior. 1991;**50**:195-203

[18] Severinsen T, Munch IC. Body core temperature during food restriction in rats. Acta Physiologica Scandinavica. 1999;**165**:299-305. DOI: 10.1046/j.1365-201x.1999.00488.x

[19] Nagashima K, Nakai S, Matsue K, et al. Effects of fasting on thermoregulatory processes and the daily oscillations in rats. The American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2003;**284**

[20] Young AA, Dawson NJ. Evidence for on-off control of heat dissipation from the tail of the rat. Canadian Journal of Physiology and Pharmacology. 1982;**60**:392-398

[21] Abe K, Kroning J, Greer MA, Critchlow V. Effects of destruction of the suprachiasmatic nuclei on the circadian rhythms in plasma corticosterone, body temperature, feeding and plasma thyrotropin. Neuroendocrinology. 1979;**29**:119-131

[22] Eastman CI, Mistlberger RE, Rechtschaffen A. Suprachiasmatic nuclei lesions eliminate circadian temperature and sleep rhythms in the rat. Physiology & Behavior. 1984;**32**:357-368

[23] Liu S, Chen XM, Yoda T, et al. Involvement of the suprachiasmatic nucleus in body temperature modulation by food deprivation in rats. Brain Research. 2002;**929**:26-36

[24] Moore RY, Eichler VB. Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. Brain Research. 1972;**42**:201-206

[25] Refinetti R, Kaufman CM, Menaker M. Complete suprachiasmatic lesions eliminate circadian rhythmicity of body temperature and locomotor activity in golden hamsters. Journal of Comparative Physiology. A. 1994;**175**:223-232

[26] Ruby NF, Dark J, Burns DE, et al. The suprachiasmatic nucleus is essential for circadian body temperature rhythms in hibernating ground squirrels. The Journal of Neuroscience. 2002;**22**:357-364

[27] Martin RD. Body temperature, activity and energy costs. Nature. 1980;**283**:335-336

[28] Refinetti R. Contribution of locomotor activity to the generation of the daily rhythm of body temperature in golden hamsters. Physiology & Behavior. 1994;**56**:829-831. DOI: 10.1016/0031-9384(94)90251-8

[29] Stirling J, Stock M. Metabolic origins of thermogenesis induced by diet. Nature. 1968;**220**:801-802. DOI: 10.1038/220801a0

[30] Ibuka N, Kawamura H. Loss of circadian rhythm in sleep-wakefulness cycle in the rat by suprachiasmatic nucleus lesions. Brain Research. 1975;**96**:76-81

[31] Balsalobre A, Brown SA, Marcacci L, et al. Resetting of circadian time in peripheral tissues by glucocorticoid signaling. Science. 2000;**289**:2344-2347. DOI: 10.1126/SCIENCE.289.5488.2344

[32] Balsalobre A, Damiola F, Schibler U. A serum shock induces circadian gene expression in mammalian tissue culture cells. Cell. 1998;**93**:929-937

[33] Damiola F, Le Minh N, Preitner N, et al. Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes & Development. 2000;**14**:2950-2961. DOI: 10.1101/GAD.183500

[34] Nagashima K, Matsue K, Konishi M, et al. The involvement of Cry1 and Cry2 genes in the regulation of the circadian body temperature rhythm in mice. American Journal of Physiology. Regulatory, Integrative and Comparative Physiology. 2005;**288**:R329-R335. DOI: 10.1152/ajpregu.00395.2004

[35] Griffin EA, Staknis D, Weitz CJ. Light-independent role of CRY1 and CRY2 in the mammalian circadian clock. Science. 1999;**286**:768-771. DOI: 10.1126/SCIENCE.286.5440.768

[36] Lucas RJ, Foster RG. Circadian clocks: A cry in the dark? Current Biology. 1999;**9**:R825-R828

[37] Stanewsky R, Kaneko M, Emery P, et al. The cryb mutation identifies cryptochrome as a circadian photoreceptor in drosophila. Cell. 1998;**95**:681-692

[38] Hoeijmakers JHJ, Horst GTJ van der, Muijtjens M, et al. Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. Nature. (1999);**398**:627-630. DOI: 10.1038/19323

[39] Bonnefont X, Albus H, Meijer JH, van der Horst GTJ. Light signalling in cryptochrome-deficient mice. Novartis Foundation Symposium. 2003;**253**:56-66-72, 102-9

[40] Peek CB, Affinati AH, Ramsey KM, et al. Circadian clock NAD+ cycle drives mitochondrial oxidative metabolism in mice. Science. 2013;**342**:123417. DOI: 10.1126/science.1243417

[41] Rutter J. Regulation of clock and NPAS2 DNA binding by the redox state of NAD cofactors suplemental online material. Science. 2001;**293**:510-515

[42] Nakahata Y, Sahar S, Astarita G, et al. Circadian control of the NAD+ salvage pathway by CLOCK-SIRT1. Science. 2009;**324**:654-657. DOI: 10.1126/science.1170803

[43] Ramsey KM, Yoshino J, Brace CS, et al. Circadian clock feedback cycle through NAMPT-mediated NAD+ biosynthesis. Science. 2009;**324**:651-654. DOI: 10.1126/science.1171641

[44] Vitaterna MH, King DP, Chang AM, et al. Mutagenesis and mapping of a mouse gene, clock, essential for circadian behavior. Science. 1994;**264**:719-725

[45] Jakus PB, Sipos K, Kispal G, Sandor A. Opposite regulation of uncoupling protein 1 and uncoupling protein 3 in vivo in brown adipose tissue of cold-exposed rats. FEBS Letters. 2002;**519**:210-214

[46] Denjean F, Lachuer J, Geloen A, et al. Differential regulation of uncoupling protein-1, −2 and −3 gene expression by sympathetic innervation in brown adipose tissue of thermoneutral or cold-exposed rats. FEBS Letters. 1999;**444**:181-185

[47] Talan MI, Kirov SA, Clow LA, Kosheleva NA. Cold acclimation-associated changes in brown adipose tissue do not necessarily indicate an increase of nonshivering thermogenesis in C57BL/6J mice. Physiology & Behavior. 1996;**60**:1285-1289

[48] Nagashima K, Nakai S, Tanaka M, Kanosue K. Neuronal circuitries involved in thermoregulation. Autonomic Neuroscience. 2000;**85**:18-25. DOI: 10.1016/S1566-0702(00)00216-2

[49] Kanosue K, Crawshaw LI, Nagashima K, Yoda T. Concepts to utilize in describing thermoregulation and neurophysiological evidence for how the system works. European Journal of Applied Physiology. 2010;**109**:5-11. DOI: 10.1007/s00421-009-1256-6

[50] Sagar SM, Sharp FR, Curran T. Expression of c-fos protein in brain: Metabolic mapping at the cellular level. Science. 1988;**240**:1328-1331

[51] Leak RK, Moore RY. Topographic organization of suprachiasmatic nucleus projection neurons. The Journal of Comparative Neurology. 2001;**433**:312-334

[52] Kalsbeek A, Palm IF, La FSE, et al. SCN outputs and the hypothalamic balance of life. Journal of Biological Rhythms. 2006;**21**:458-469

[53] Yoshida K, Konishi M, Nagashima K, et al. Fos activation in hypothalamic neurons during cold or warm exposure: Projections to periaqueductal gray matter. Neuroscience. 2005;**133**:1039-1046. DOI: 10.1016/j.neuroscience.2005.03.044

[54] Freeman PH, Wellman PJ. Brown adipose tissue thermogenesis induced by low level electrical stimulation of hypothalamus in rats. Brain Research Bulletin. 1987;**18**:7-11. DOI: 10.1016/0361-9230(87)90026-8

[55] Amir S. Stimulation of the paraventricular nucleus with glutamate activates interscapular brown adipose tissue thermogenesis in rats. Brain Research. 1990;**508**:152-155. DOI: 10.1016/0006-8993(90)91129-5

[56] Yoshimatsu H, Egawa M, Bray GA. Sympathetic nerve activity after discrete hypothalamic injections of l-glutamate. Brain Research. 1993;**601**:121-128. DOI: 10.1016/0006-8993(93)91702-T

[57] Nakamura K, Morrison SF. A thermosensory pathway that controls body temperature. Nature Neuroscience. 2008;**11**:62-71. DOI: 10.1038/nn2027

[58] Wang L, Martinez V, Barrachina MDTY. Fos expression in the brain induced by peripheral injection of CCK or leptin plus CCK in fasted lean mice. Brain Research. 1998;**791**:157-166. DOI: 10.1016/S0006-8993(98)00091-2

[59] Morikawa Y, Ueyama E, Senba E. Fasting-induced activation of mitogen-activated protein kinases (ERK/p38) in the mouse hypothalamus. Journal of Neuroendocrinology. 2004;**16**:105-112

[60] Morton GJ, Cummings DE, Baskin DG, et al. Central nervous system control of food intake and body weight. Nature. 2006;**443**:289-295. DOI: 10.1038/nature05026

[61] Saeb-Parsy K, Lombardelli S, Khan FZ, et al. Neural connections of hypothalamic neuroendocrine nuclei in the rat. Journal of Neuroendocrinology. 2000;**12**:635-648

[62] Van der Beek EM, Horvath TL, Wiegant VM, et al. Evidence for a direct neuronal pathway from the suprachiasmatic nucleus to the gonadotropin-releasing hormone system: Combined tracing and light and electron microscopic immunocytochemical studies. The Journal of Comparative Neurology. 1997;**384**:569-579

[63] Yi CX, van der Vliet J, Dai J, et al. Ventromedial arcuate nucleus communicates peripheral metabolic information to the suprachiasmatic nucleus. Endocrinology. 2006;**147**:283-294

[64] Luque RM, Park S, Kineman RD. Severity of the catabolic condition differentially modulates hypothalamic expression of growth hormone-releasing hormone in the fasted mouse: Potential role of neuropeptide Y and corticotropin-releasing hormone. Endocrinology. 2007;**148**:300-309. DOI: 10.1210/en.2006-0592

[65] Freeman DA, Lewis DA, Kauffman AS, et al. Reduced leptin concentrations are permissive for display of torpor in Siberian hamsters. American Journal of Physiology. Regulatory, Integrative and Comparative Physiology. 2004;**287**:R97-R103. DOI: 10.1152/ajpregu.00716.2003

[66] Gluck EF, Stephens N, Swoap SJ. Peripheral ghrelin deepens torpor bouts in mice through the arcuate nucleus neuropeptide Y signaling pathway. American Journal of Physiology.

Regulatory, Integrative and Comparative Physiology. 2006;**291**:R1303-R1309. DOI: 10.1152/ajpregu.00232.2006

[67] Morton GJ, Schwartz MW. The NPY = AgRP neuron and energy homeostasis. International Journal of Obesity. 2001;**25**:56-62

[68] Mercer JG, Hoggard N, Williams LM, et al. Localization of leptin receptor mRNA and the long form splice variant (Ob-Rb) in mouse hypothalamus and adjacent brain regions by in situ hybridization. FEBS Letters. 1996;**387**:113-116

[69] Willesen MG, Kristensen P, Rømer J. Co-localization of growth hormone secretagogue receptor and NPY mRNA in the arcuate nucleus of the rat. Neuroendocrinology. 1999;**70**:306-316

[70] Guan XM, Hess JF, Yu H, et al. Differential expression of mRNA for leptin receptor isoforms in the rat brain. Molecular and Cellular Endocrinology. 1997;**133**:1-7

[71] Zigman JM, Jones JE, Lee CE, et al. Expression of ghrelin receptor mRNA in the rat and the mouse brain. The Journal of Comparative Neurology. 2006;**494**:528-548. DOI: 10.1002/cne.20823

[72] Inyushkin AN, Bhumbra GS, Dyball REJ. Leptin modulates spike coding in the rat suprachiasmatic nucleus. Journal of Neuroendocrinology. 2009;**21**:705-714. DOI: 10.1111/j.1365-2826.2009.01889.x

[73] Yannielli PC, Molyneux PC, Harrington ME, Golombek DA. Ghrelin effects on the circadian system of mice. The Journal of Neuroscience. 2007;**27**:2890-2895. DOI: 10.1523/JNEUROSCI.3913-06.2007

[74] Tokizawa K, Onoue Y, Uchida Y, Nagashima K. Ghrelin induces time-dependent modulation of thermoregulation in the cold. Chronobiology International. 2012;**29**:736-746. DOI: 10.3109/07420528.2012.678452

[75] Mano-Otagiri A, Ohata H, Iwasaki-Sekino A, et al. Ghrelin suppresses noradrenaline release in the brown adipose tissue of rats. The Journal of Endocrinology. 2009;**201**:341-349. DOI: 10.1677/JOE-08-0374

[76] Yasuda T, Masaki T, Kakuma T, Yoshimatsu H. Centrally administered ghrelin suppresses sympathetic nerve activity in brown adipose tissue of rats. Neuroscience Letters. 2003;**349**:75-78. DOI: 10.1016/S0304-3940(03)00789-4

[77] Gamble KL, Ehlen JC, Albers HE. Circadian control during the day and night: Role of neuropeptide Y Y5 receptors in the suprachiasmatic nucleus. Brain Research Bulletin. 2005;**65**:513-519. DOI: 10.1016/j.brainresbull.2005.03.004

Chapter 3

Sex and Sex Hormones in Tissue Homeostasis

Judith Lechner and Gerhard Gstraunthaler

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76177

Abstract

Women are not small men. Sex-specific differences do not only affect the classical target organs of sexual differentiation and reproduction, but have been found to involve most, if not all the organs and tissues in the body. One of the consequences of this dimorphism is that diseases manifest in a sex- and gender-specific way. Key to maintenance of a healthy state is functioning tissue able to cope with insults. Regulated death of damaged cells and replacement with new cells by proliferation is a prerequisite for maintaining tissue function taking place at different pace in the different organs. The intent of this chapter is to review current evidence for sex-specific differences in tissue homeostasis focusing on the variability of hormone exposure characteristic for the female reproductive life stages.

Keywords: tissue maintenance, sex differences, proliferation, cell death, kidney, menstrual cycle

1. Introduction

Living systems are continuously challenged by potentially toxic internal and external processes. The normal metabolic function of the cells produces a plethora of potentially damaging oxidative metabolites inducing damage in DNA, proteins, and lipids. In addition, living cells are exposed to a variety of external factors, which may be internalized as building blocks and/or energy sources. These vital processes put the organism at risk to be harmed. Coping strategies are necessary to avoid damage. There are several lines of cellular defenses induced via cell stress pathways, including compartmentalization processes, enzymatic modification, externalization, degradation, and repair [1, 2].

Ultimately, these processes may not be sufficient to prevent major cellular damage. Therefore, every cell is in addition equipped with internal cell death programs, which can be activated in order to prevent a damaged cell to cause harm to the organism [3]. Cell losses are inevitable

and take place continuously in our bodies even without a specific trigger. The rate of cell death may be significantly enhanced at times of increased challenges. Cellular losses are necessary in order to prevent detrimental effects like neoplastic transformation [4]. Thus, programmed cell death needs to be carefully balanced, and this ability is a key determinant for the health and survival of the organism.

Cells lost by cell death need to be replaced in order to maintain cell numbers and ultimately tissue function. Controlled regeneration is, thus, required to cope for cell losses due to toxic challenges derived from internal and external sources, be they derived from normal metabolic processes or damaging environmental stimuli. Cell losses and proliferation need to be carefully balanced in order to guarantee proper function. Thus, the process of tissue homeostasis, that is, the capability to send damaged cells into cell death programs, to replace the cells by proliferation, and to regulate the exact balance of these events are crucial processes for preserving a healthy state. Any distortion of the balance between cell death and cell proliferation—be it by overwhelming damaging events beyond the host's range of tolerance and/or primarily ineffective or maladaptive homeostatic mechanisms by the host—is prone to induce malfunctioning of the organs in the body ultimately causing disease and potentially death.

2. Sex and sex hormones in tissue homeostasis

2.1. Origin of sex differences

During development, sex differences originate from genetic and hormonal influences. Master regulators for male sex differentiation, like SRY, are encoded by DNA of the Y chromosome governing the embryonic development of the male phenotype in mammals. Female or male gonadal development gives rise to a sex-specific hormonal environment [5]. Sex hormones induce organizational effects during the life span causing persistent sex-specific changes within the tissues, for example, by epigenetic modifications [6, 7]. Activational effects further introduce sex differences in tissue structure and function depending on the pattern of exposure to gonadal hormones. All the organs in the body are affected throughout life [8]. In this respect, sex differences are based on the different chromosomal equipment that qualifies every cell in the body as male or female. These basic differences are further shaped by sex hormones depending on previous or current, transient or persistent exposure [9]. This hypothesis was phrased by Arnold [10] as follows: "XX and XY cells are different prior to the secretion of gonadal hormones, and gonadal hormones affect XX and XY cells unequally."

Sex hormones act through receptors widely expressed throughout the cells of the body. The classical estrogen (ERα, ERβ), androgen (AR) and progesterone (PR) receptors belong to the nuclear receptor protein family acting through the nucleus as transcription factor or co-factor. In addition to their nuclear actions, they were found to be localized to the cell membrane and mitochondria inducing fast, non-genomic intracellular signaling pathways, for example, by the interaction with growth factor or cytokine receptors. Estrogen-binding cell membrane-localized receptors of the seven transmembrane receptor family were also characterized, for example, G-protein coupled estrogen receptor, GPR30/GPER-1 [11–13].

Ultimately, differences between the sexes derive from chromosomal and hormonal sex differences, which are further influenced by environmental factors. Thus, differences originating from the biological sex are further shaped by gender, which refers to the perceptions of male or female identity and depends on sex-based social structures [8].

2.2. Influence of sex and sex hormones on cellular proliferation

Tissue homeostasis is guaranteed, when cells lost in physiological tissue turnover or under stress conditions are replaced by proliferation. Organs with high demanding functions have increased regeneration potential and continually renew their cell populations. This is the case for intestine, skin, and blood, for example. Liver, bone, and blood even have the capacity to fully recover to the original size after loss of tissue [14]. Other organs have lower regeneration potential, like the heart, brain, and kidney [15–17]. Many organs contain stem cell niches hosting adult tissue stem cells that are precursor cells maintained in a relatively undifferentiated state ready to replace lost cells by proliferation followed by differentiation [18, 19].

Sex hormones have classically been implicated in regulation of proliferation of cells of reproductive organs and cancer of reproductive tissue [20–24]. Besides these effects, sex hormones were also found to have pronounced effects on the proliferation of different stem cell populations. Cell proliferation of embryonic stem (ES) cells was found to be enhanced by female gonadal hormones [25]. ES cells are derived from the inner cell mass of the embryoid body. They can self-renew in vitro and are pluripotent, that is, they can differentiate into all the cell types of the body [26]. Estrogen appears to act via nuclear and cell surface signaling pathways involving Erk1/2 activation, cyclin-dependent kinases and proto-oncogenes like c-myc, c-fos, c-jun, and pRB in ES cells. In addition, store-operated calcium channels were found to play a role in estrogen-mediated cell proliferation through the transcription factor NF-AT [27].

Differentiation of ES cells into dopaminergic neurons was also shown to be affected by estrogen. ERβ promoted differentiation by crosstalk signaling with insulin like growth factor-1 [28]. Motor neuron differentiation from ES cells was found to be enhanced by 17-β estradiol and progesterone through nuclear ERα and progesterone receptor [29]. Dopaminergic precursors derived from ES cells were found to increase proliferation upon treatment with progesterone in vitro [30].

Induced pluripotent stem (iPS) cells are similar to ES cells with regard to their ability to differentiate into all cell types, providing a promising tool for in vitro research and regenerative medicine. They are derived from adult mature cells by reprogramming through the introduction of specific transcription factors [31]. Similar to ES cells, sex hormones were shown to affect iPS cells. Neuronal cells derived by differentiation of iPS cells showed increased dendritic branching by treatment with 17-β estradiol [32]. Functional integration of dopaminergic neuronal cells from iPS cells into neuronal circuits was found to be enhanced by estradiol [33]. Testosterone was described to enhance differentiation of iPS cells into insulin-producing cells [34].

Sex differences were also described for tissue stem cells in vivo. Adult stem cells are believed to provide a local pool of self-renewing, multipotent cells pivotal in tissue homeostasis and

recovery upon damage [35]. Stem cells in many stem cell niches appear to have a higher ability to self-renew, have an increased regeneration potential, and in some cases, show higher proliferative activity in women [36, 37]. Intrinsic sexual dimorphism was described for neural stem cells that hold much promise for potential brain damage repair therapy in the future. Proliferation of neural stem cell was, for example, shown to depend on hormone changes in the adult mouse due to the estrous cycle, pregnancy, reproductive status, and age. Phases of high estrogen exposure like pro-estrus were found to be associated with increased hippocampal adult neurogenesis indicating a role of estrogens [38]. Differential expression of sex steroid receptors and androgen metabolizing enzymes may result in differential outcomes in neural stem cell transplantation [39]. Neural stem cell proliferation was found to be dependent on nuclear ERs, while oligodendroglial differentiation was stimulated by cell membrane-associated ERs [40]. Other researcher also proposed that actions of sex steroids on the brain might be correlated with reduced brain damage. Intact females were found to be less susceptible upon injury than ovariectomized females and males [41]. Similarly, muscle-derived stem cells derived from female mice and transplanted into dystrophic mutant mice showed a better potential to regenerate skeletal muscle than stem cells from males [42].

Hematopoietic stem cells were found to be more abundant and proliferative in female mice in comparison to males dependent on estrogen exposure [43]. 17-β estradiol was found to improve hematopoietic differentiation from human iPS cells and from human umbilical cord blood through ERα signaling suggesting a universal function for estrogen in hematopoietic stem cell differentiation [44, 45].

Estrogens have beneficial effects on bone regeneration [46]. Osteoblasts are stimulated by estrogen to proliferate with distinct roles for ERα and ERβ [47]. In vitro, proliferation of bone marrow mesenchymal stromal cells was found to be enhanced by estrogen [48]. Estrogens enhanced the proliferation and migration of bone marrow-derived endothelial progenitor cells to ischemic regions of the heart facilitating repair and regeneration [49]. Androgens were also described to stimulate the proliferation and angiogenesis/vascular repair capability of circulating endothelial progenitor cells in males, not females [50].

2.3. Sex differences in cell survival

When progenitor cells involved in tissue regeneration enter a cell senescence state, tissue homeostasis may be compromised. The cells are able to permanently halt the cell cycle and persist in a quiescent, but still functional state [51]. This is a possible fate of cells damaged beyond repair. The three major types of senescence are replicative senescence, oncogene-induced senescence, and DNA damage-induced DNA damage. The DNA damage response pathway appears to be eventually involved in the execution of the program independent of the primary stimulus [52]. Furthermore, senescent cells are able to influence their neighboring cells by secretion of a range of activating signals referred to as senescence-associated secretory phenotype. The signals may favor a pro-inflammatory or—alternatively—an immunosuppressive/pro-fibrotic state. Both phases appear to be important for successful tissue repair and the timing of the shift in the secretome might be crucial [53]. The etiology and progression of

many cancerous or age-related diseases have been shown to be influenced by the secretome of senescence cells [54–56].

Alternatively, cells may activate a cell death program as a means to ensure physiological tissue renewal or in response to overwhelming damage. The most common are type I cell death programs or apoptosis, type II or autophagy, type III or necrosis, and mitotic catastrophe [57, 58]. The cell death modalities are characterized by different morphological criteria and are executed by specific intracellular signaling cascades. Specific catabolic enzymes are typically associated with specific forms of cell death, for example, caspases with apoptosis. The pathways are interdependent. The intensity of the damage signal is often decisive for the type of cell death program that is executed or the switch from one modality to the other. In addition, autophagy is not primarily regarded as a cell death mechanism. Autophagy describes a process involving the break-down and recycling of specific subcellular organelles. This process may provide a cell survival strategy by reducing damaged organelles and/or shifting internal resources in order to optimize cell survival. Only if the damaging process exceeds the cellular defenses, cells die in the process [59, 60].

Regarding the role of sex in cell fate decisions, several reports have highlighted distinct sex-dependent differences. Sex hormones have been shown to influence the propensity of cells to undergo apoptosis. In general, lower concentrations of estrogen were found to be protective, while higher concentrations were found to promote apoptosis. Androgens were found to enhance, but also to suppress apoptosis depending on the cellular context [61, 62]. For example, estrogen and testosterone were described to reduce apoptosis in skeletal muscle cells [63]. Both hormones also appear to prevent apoptosis in neuronal cells adding to their neuroprotective function [64, 65]. An anti-apoptotic action of testosterone was also described in pancreatic β cells from male rats, but not from female rats [66]. Estrogen and estrogenic compounds, however, appeared to enhance apoptosis in pancreatic β cells in elderly mice, while it reduced apoptosis in young animals [67]. Regarding vascular endothelial cells, several studies have shown that estrogens protect from apoptotic cell death [68, 69], while apoptosis increased in coronary artery endothelia from postmenopausal women [70]. Testosterone was found to induce apoptosis in endothelial cells [71–73]. Treatment with testosterone also induced apoptosis or senescence in human dermal papilla cells, a process implied in inherited male alopecia [74, 75]. In addition, androgens were found to promote apoptosis in renal and intestinal cell lines and bone marrow-derived macrophages [76–78].

Overall, sex hormones appear to influence cell fate decisions depending on the cell context. Hormone independent sex differences are also apparent shaping the cellular response [79]. Thus, female and male cells appear to rely on different coping strategies in response to stressors. For example, vascular smooth muscle cells isolated from aorta of male rats appear to be more inclined to undergo apoptosis in response to UV irradiation, while female cells are more prone to execute the cell senescence program [62, 80]. Female cells showed characteristics of autophagy, which is presumed to help female cells to repair the UV-induced intracellular damages ultimately providing a survival strategy [81]. In addition, female cells were found to better adhere to the growth support, thus avoiding apoptotic cell death initiation by cell detachment, a process called anoikis-resistance. Differences in the intracellular organization

of the actin cytoskeleton and increased phosphorylation of focal adhesion kinase were attributed to this higher propensity of female cells to adhere [81]. Apparently, female cells are better equipped to prevent cell death. While autophagic processes were found to protect neuronal cells from cell death due to starving in female rats, male cells were not able to benefit and died more often from autophagic cell death [82]. Organ-specific sex differences were found in constitutive autophagy, a process implicated in physiological tissue turnover. While autophagic marker proteins were increased in the male versus female heart and liver, no such differences were observed in the kidneys [83]. Osteoblasts showed reduced autophagy in aging female mice, while the rate remained constant in males over the life span. This was correlated with higher oxidative stress in female cells, thus potentially enhancing bone loss and playing a role in the pathophysiology of osteoporosis in women [84]. Estrogens alter the redox balance and counteract bone loss [46]. Stem cells involved in generation of osteoblast, namely bone marrow derived mesenchymal stem cells, were found to be influenced by estrogens not only inducing increased proliferation, but also reducing senescence and apoptosis [85].

In general, increased antioxidative cellular defenses were implicated to provide females with better strategies to cope with oxidative stress and prevent cellular losses [62]. Differences in basal redox state and responses to oxidative imbalance were demonstrated between female and male cells [86]. For example, female cells were shown to produce less hydrogen peroxide and superoxide anion. Anti-oxidative enzymes, such as superoxide dismutase (SOD) and catalase, showed higher basic activity in female versus male cells [87]. Thioredoxin reductases and manganese SOD were increased by estrogen in cardiomyocytes [88, 89]. In vascular smooth muscle cells and circulating monocytes, estrogen was found to stimulate manganese and extracellular SOD expression [90]. Estrogen was, furthermore, shown to modulate the expression of other key molecular defense enzymes differently in XX and XY cells, for example, poly-ADP ribose polymerase (PARP), a DNA damage repair enzyme, or RLIP76, a cell-protective transporter protein [86].

PARP was also found to play a major role in sex differences in stroke. Experiments in mice have shown that ischemic neuronal cell death is dependent on intact neuronal nitric oxide synthase (nNOS)/PARP signaling, while in females a protection is provided by estrogen paradoxically also requiring an intact nNOS/PAPR axis [91]. While male neuronal cells appear to die via a PARP-mediated caspase independent pathway, ischemic cell death pathways appear to be dependent on activation of caspase-dependent cell death pathways in females [9, 92]. Such sex-specific differences may be relevant for the sex-specific difference in stroke prevalence [93, 94].

PARP signaling was also implied in sex differences in cell fate decisions in kidney cells. In a mouse model of immune-mediated nephritis, PARP signaling induced necrosis in male cells and inhibition of PARP shifted the pro-inflammatory necrotic cell death to an anti-inflammatory apoptotic pathway. In female cells, by contrast, cell death was independent of PARP and female cells preferentially underwent apoptosis. Estrogen acted in a pro-survival manner in female cells only. In addition to the kidney cells, bone marrow-derived hematopoietic cells showed similar sex differences [95].

Mitochondria play a crucial role in apoptotic cell death programs. Estrogens were described to modulate the propensity for mitochondrial initiation of apoptosis [61, 96]. Estrogen-mediated

modulation of mitochondrial function is achieved by hormone effects on the expression of mitochondrial and nuclear genome-encoded mitochondrial proteins [97–101]. Since mitochondria are central in the cellular defense against oxidative stress, mitochondria are especially sensitive to accumulate damage over time. Malfunctioning mitochondria accumulate during aging, a process regarded as a major contributor to the onset of many age-related diseases [102, 103]. Sex differences were observed in this process. Delayed malfunctioning of mitochondria during the aging process might provide females with better strategies to cope with cellular stressors. Maternal transmission of mitochondria appears to provide a more favorable environment in female offspring [104]. Xist, an RNA-coding gene involved in X chromosome inactivation in female cells, appears to be pivotal for mitochondrial maintenance [105]. Mitochondrial biogenesis and degradation by mitophagy are dependent on the transcription factors p53 and FOXO [106, 107]. Sex-specific differences in the activity of these nuclear factors were reported. Males were shown to exhibit relatively greater FOXO activity. Females, on the other hand, had higher p53 activity resulting in sex-specific differences in the ability to maintain healthy mitochondrial functionality during aging [105].

2.4. Potential consequences of sex-specific differences in tissue homeostasis

The abovementioned paragraphs have described examples of sex-specific differences regarding processes involved in tissue maintenance, like the control of cell proliferation and cell death. Such effects may ultimately result in differences in the ability of female and male tissues to cope with stressors affecting the ability to repair and restore function or develop disease. Many diseases show different incidence and prevalence rates in men and women derived from sex and gender specific pathophysiological mechanisms. Sex and gender differences have been studied intensively in the neural system, the cardiovascular system, and the development of cancer, among others [108–110].

Mechanisms underlying differences in kidney diseases between men and women are less well known, despite renal diseases with a high morbidity and mortality risk being a challenging problem for patients, clinicians and society [111, 112]. International registries show that fewer women than men develop kidney failure [113–116]. The underlying causes, however, are widely unknown. The presumed female protective effects appear to be most pronounced in women of reproductive age [117–120]. This finding suggests that female sex hormones might play a key role. Estrogen was proposed to be renoprotective via modulating renal perfusion and effects on the vasculature. Furthermore, a role of estrogen was proposed in the control of the local renal renin-angiotensin system [121–123]. On the other hand, estrogen was implicated in the control of mesangial and tubular cell proliferation and linked to neoplastic transformation of the kidney in hamster kidneys. Low estrogen concentrations were shown to induce proliferation in glomerular mesangial cell, while high concentrations suppressed it [124]. Primary proximal tubular cell explants and subcultured dissociated proximal tubular cells were shown to proliferate, when treated with estrogen at physiologic concentrations [125]. This finding was confirmed in primary rabbit proximal tubular cells, which showed increased proliferation upon estrogen treatment [126].

We have previously shown that renal tubular cell-specific proteins appear at higher rates in the urine of healthy women at specific hormonal transition phases of the natural ovulating

menstrual cycle. Urinary samples from healthy probands showed increased rates of urinary excretion of the marker proteins Fructose-1,6-bisphosphatase and Glutathione-S-transferase α, when estrogen levels decreased after a preceding height associated with ovulation and luteal phase [127]. Both enzymes are specifically found in proximal tubular cells, the most populous cell type in the kidney. When proximal tubular cells are damaged, intracellular enzymes are released into the urine, making them clinical markers for kidney injury. In contrast to ovulating women, male probands and postmenopausal women showed consistently low levels of these renal marker proteins over time. Other urinary proteins, for example, albumin, α1-microglobulin, and immunglobulin G, which are markers for functional changes of the glomerular filter and/or tubular protein resorption, showed constant urinary excretion suggesting that the observed increases of proximal tubular marker protein release in ovulating women are not accompanied by major functional distress of the kidneys [127]. This pattern of urinary marker proteins excretion suggests that cyclical changes of female hormones might affect kidney cell health. Tubular enzymes are released into the urine, if proximal tubular cells are sloughed off and/or their plasma membranes become leaky. This could be due to tubular cells being transiently more prone to damage in situ resulting in plasma membrane leakage or to the cells being removed from the tubular epithelium and released into the urinary space, for example, by apoptosis. Both processes lead to increased cell losses. Tissue homeostasis would be maintained, if increased cell removal was accompanied by increased cell proliferation. This could be the case during the high estrogen exposure phases preceding the observed tubular enzyme releases into the urine. The finding that tubular cells are able to proliferate upon estrogen treatment [126] is in line with this hypothesis. Such a periodic interplay between cell proliferation and cell loss brought about by the specific changes in the pattern of sex hormone exposure might result in an increased rate of tissue renewal. If this was the case, then women in their reproductive years would possess an efficient means to easily get rid of potentially injured, dysfunctional or simply older proximal tubular cells by replacing them with fresh new cells. Such a transiently increased repair capacity might provide an efficient means to cyclically renew renal tubular tissue leading to a higher resistance to damage. It is, however, also possible that during the short phases of increased tubular cell death, the kidneys might be especially sensitive to damage. With regard to the potential beneficial action of treatment of renal proximal tubular cells with a proliferation-inducing growth factor, we have previously demonstrated that epidermal growth factor (EGF) treatment was able to accelerate tissue repair after treatment with interferon α (IFNα) in vitro. However, if EGF was present before or during IFNα treatment, epithelial barrier destabilization was intensified [128, 129]. Therefore, the overall effect might be different in other cycle contexts or under hormone therapy, if the vulnerable phases might not be restricted to short periods.

3. Conclusion

In conclusion, it appears that males and females are equipped with stress coping strategies that may differ between the sexes. Sex differences have been demonstrated in the cellular

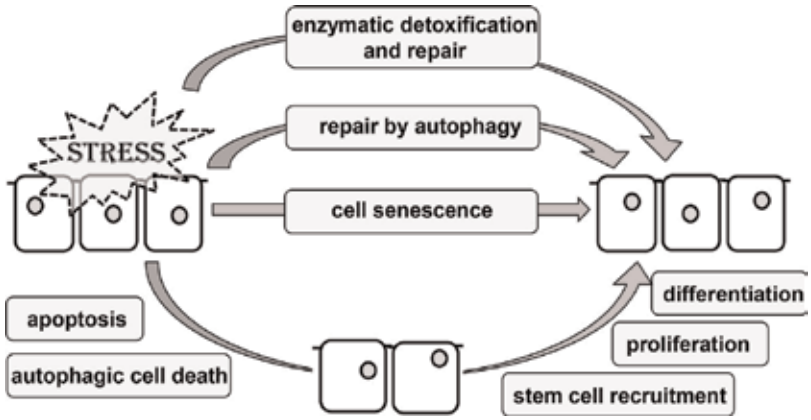

Figure 1. Sex and sex hormone-induced differences in tissue homeostasis. The figure shows strategies involved in tissue maintenance following cellular stress. Sex differences and sex hormone-dependent effects have been shown in these processes in different organs and tissues.

expression levels and activity of detoxifying and repair enzymes, in the propensity to use autophagic processes for repair, in senescence or cell death programs and in the ability to replace cells by proliferation (**Figure 1**). These effects are apparent in isolated cells and are further shaped by exposure to sex hormones. Sex hormone levels cyclically changing in dependence of the female reproductive hormone cycle might enhance physiological tissue regeneration and provide greater damage repair potential. Overall, female tissues appear to be more resistant to cellular stress than their male counterparts.

Acknowledgements

This work was supported by the Austrian Central Bank Fund project No 13671 to J Lechner.

Conflict of interest

The authors confirm that there are no conflicts of interest.

Author details

Judith Lechner* and Gerhard Gstraunthaler

*Address all correspondence to: judith.lechner@i-med.ac.at

Division of Physiology, Medical University of Innsbruck, Innsbruck, Austria

References

[1] Fulda S, Gorman AM, Hori O, Samali A. Cellular stress responses: Cell survival and cell death. International Journal of Cell Biology. 2010;**2010**:23

[2] Jennings P. Stress response pathways, toxicity pathways and adverse outcome pathways. Archives of Toxicology. 2013;**87**(1):13-14

[3] Lockshin RA, Zakeri Z. Cell death in health and disease. Journal of Cellular and Molecular Medicine. 2007;**11**(6):1214-1224

[4] Zhivotovsky B, Orrenius S. Carcinogenesis and apoptosis: Paradigms and paradoxes. Carcinogenesis. 2006;**27**(10):1939-1945

[5] Quinn A, Koopman P. The molecular genetics of sex determination and sex reversal in mammals. Seminars in Reproductive Medicine. 2012;**30**(5):351-363

[6] Wang Y, Liu H, Sun Z. Lamarck rises from his grave: Parental environment-induced epigenetic inheritance in model organisms and humans. Biological Reviews. 2017;**92**(4): 2084-2111

[7] Milagre I, Stubbs TM, King MR, Spindel J, Santos F, Krueger F, Bachman M, Segonds-Pichon A, Balasubramanian S, Andrews SR, Dean W, Reik W. Gender differences in global but not targeted demethylation in iPSC reprogramming. Cell Reports. 2017;**18**(5): 1079-1089

[8] Regitz-Zagrosek V, Kararigas G. Mechanistic pathways of sex differences in cardiovascular disease. Physiological Reviews. 2017;**97**(1):1-37

[9] Siegel C, Turtzo C, McCullough LD. Sex differences in cerebral ischemia: Possible molecular mechanisms. Journal of Neuroscience Research. 2010;**88**(13):2765-2774

[10] Arnold AP. The organizational–activational hypothesis as the foundation for a unified theory of sexual differentiation of all mammalian tissues. Hormones and Behavior. 2009; **55**(5):570-578

[11] Arnal JF, Lenfant F, Metivier R, Flouriot G, Henrion D, Adlanmerini M, Fontaine C, Gourdy P, Chambon P, Katzenellenbogen B, Katzenellenbogen J. Membrane and nuclear estrogen receptor alpha actions: From tissue specificity to medical implications. Physiological Reviews. 2017;**97**(3):1045-1087

[12] Banerjee S, Chambliss KL, Mineo C, Shaul PW. Recent insights into non-nuclear actions of estrogen receptor alpha. Steroids. 2014;**81**:64-69

[13] Boese AC, Kim SC, Yin KJ, Lee JP, Hamblin MH. Sex differences in vascular physiology and pathophysiology: Estrogen and androgen signaling in health and disease. American Journal of Physiology—Heart and Circulatory Physiology. 2017;**313**(3):H524-Hh45

[14] Lindahl A. Chapter 3—Tissue Homeostasis. Tissue Engineering. Burlington: Academic Press; 2008. pp. 73-87

[15] Ellison GM, Nadal-Ginard B, Torella D. Optimizing cardiac repair and regeneration through activation of the endogenous cardiac stem cell compartment. Journal of Cardiovascular Translational Research. 2012;**5**(5):667-677

[16] Lin R, Iacovitti L. Classic and novel stem cell niches in brain homeostasis and repair. Brain Research. 2015;**1628**(Pt B):327-342

[17] Park HC, Yasuda K, Kuo MC, Ni J, Ratliff B, Chander P, Goligorsky MS. Renal capsule as a stem cell niche. American Journal of Physiology—Renal Physiology. 2010;**298**(5):F1254-F1262

[18] Rojas-Rios P, Gonzalez-Reyes A. Concise review: The plasticity of stem cell niches: A general property behind tissue homeostasis and repair. Stem Cells (Dayton, Ohio). 2014; **32**(4):852-859

[19] Wabik A, Jones PH. Switching roles: The functional plasticity of adult tissue stem cells. The EMBO Journal. 2015;**34**(9):1164-1179

[20] Fox EM, Andrade J, Shupnik MA. Novel actions of estrogen to promote proliferation: Integration of cytoplasmic and nuclear pathways. Steroids. 2009;**74**(7):622-627

[21] Bluemn EG, Nelson PS. The androgen/androgen receptor axis in prostate cancer. Current Opinion in Oncology. 2012;**24**(3):251-257

[22] Maximov PY, Abderrahman B, Curpan RF, Hawsawi YM, Fan P, Jordan VC. A unifying biology of sex steroid-induced apoptosis in prostate and breast cancers. Endocrine-related Cancer. 2018;**25**(2):R83-r113

[23] Russo J, Russo IH. The role of estrogen in the initiation of breast cancer. The Journal of Steroid Biochemistry and Molecular Biology. 2006;**102**(1–5):89-96

[24] Finlay-Schultz J, Sartorius CA. Steroid hormones, steroid receptors, and breast cancer stem cells. Journal of Mammary Gland Biology and Neoplasia. 2015;**20**(1–2):39-50

[25] Han HJ, Heo JS, Lee YJ. Estradiol-17beta stimulates proliferation of mouse embryonic stem cells: Involvement of MAPKs and CDKs as well as protooncogenes. American Journal of Physiology—Cell Physiology. 2006;**290**(4):C1067-C1075

[26] Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. Science (New York, NY). 1998;**282**(5391):1145

[27] Wong CK, So WY, Law SK, Leung FP, Yau KL, Yao X, Huang Y, Li X, Tsang SY. Estrogen controls embryonic stem cell proliferation via store-operated calcium entry and the nuclear factor of activated T-cells (NFAT). Journal of Cellular Physiology. 2012;**227**(6): 2519-2530

[28] Li H, Ding C, Ding Z, Ling M, Wang T, Wang W, Huang B. 17β-Oestradiol promotes differentiation of human embryonic stem cells into dopamine neurons via cross-talk between insulin-like growth factors-1 and oestrogen receptor β. Journal of Cellular and Molecular Medicine. 2017;**21**(8):1605-1618

[29] Lopez-Gonzalez R, Camacho-Arroyo I, Velasco I. Progesterone and 17beta-estradiol increase differentiation of mouse embryonic stem cells to motor neurons. IUBMB Life. 2011;**63**(10):930-939

[30] Díaz NF, Díaz-Martínez NE, Velasco I, Camacho-Arroyo I. Progesterone increases dopamine neuron number in differentiating mouse embryonic stem cells. Journal of Neuroendocrinology. 2009;**21**(8):730-736

[31] Yamanaka S. Induced pluripotent stem cells: Past, present, and future. Cell Stem Cell. 2012;**10**(6):678-684

[32] Shum C, Macedo SC, Warre-Cornish K, Cocks G, Price J, Srivastava DP. Utilizing induced pluripotent stem cells (iPSCs) to understand the actions of estrogens in human neurons. Hormones and Behavior. 2015;**74**:228-242

[33] Nishimura K, Doi D, Samata B, Murayama S, Tahara T, Onoe H, Takahashi J. Estradiol facilitates functional integration of iPSC-derived dopaminergic neurons into striatal neuronal circuits via activation of integrin α5β1. Stem Cell Reports. 2016;**6**(4):511-524

[34] Liu H, Guo D, Ruzi A, Chen Y, Pan T, Yang F, Li J, Xu K, Zhou T, Qin D, Li YX. Testosterone improves the differentiation efficiency of insulin-producing cells from human induced pluripotent stem cells. PLoS One. 2017;**12**(6):e0179353

[35] Tower J. Stress and stem cells. Wiley Interdisciplinary Reviews—Developmental Biology. 2012;**1**(6):789-802

[36] Dulken B, Brunet A. Stem cell aging and sex: Are we missing something? Cell Stem Cell. 2015;**16**(6):588-590

[37] Ray R, Novotny NM, Crisostomo PR, Lahm T, Abarbanell A, Meldrum DR. Sex steroids and stem cell function, Molecular Medicine (Cambridge, Mass). 2008;**14**(7–8):493-501

[38] Pawluski JL, Brummelte S, Barha CK, Crozier TM, Galea LAM. Effects of steroid hormones on neurogenesis in the hippocampus of the adult female rodent during the estrous cycle, pregnancy, lactation and aging. Frontiers in Neuroendocrinology. 2009; **30**(3):343-357

[39] Lecanu L. Sex, the underestimated potential determining factor in brain tissue repair strategy. Stem Cells and Development. 2011;**20**(12):2031-2035

[40] Okada M, Makino A, Nakajima M, Okuyama S, Furukawa S, Furukawa Y. Estrogen stimulates proliferation and differentiation of neural stem/progenitor cells through different signal transduction pathways. International Journal of Molecular Sciences. 2010; **11**(10):4114-4123

[41] Helen M, Bramlett W, Dalton Dietrich. Neuropathological protection after traumatic brain injury in intact female rats versus males or ovariectomized females. Journal of Neurotrauma 2001;**18**(9):891-900

[42] Deasy BM, Schugar RC, Huard J. Sex differences in muscle-derived stem cells and skeletal muscle. Critical Reviews in Eukaryotic Gene Expression. 2008;**18**(2):173-188

[43] Nakada D, Oguro H, Levi BP, Ryan N, Kitano A, Saitoh Y, Takeichi M, Wendt GR, Morrison SJ. Oestrogen increases haematopoietic stem-cell self-renewal in females and during pregnancy. Nature. 2014;**505**:555

[44] Kim HR, Lee JH, Heo HR, Yang SR, Ha KS, Park WS, Han ET, Song H, Hong SH. Improved hematopoietic differentiation of human pluripotent stem cells via estrogen receptor signaling pathway. Cell & Bioscience. 2016;**6**(1):50

[45] Heo HR, Chen L, An B, Kim KS, Ji J, Hong SH. Hormonal regulation of hematopoietic stem cells and their niche: A focus on estrogen. International Journal of Stem Cells. 2015; **8**(1):18-23

[46] Manolagas SC. Steroids and osteoporosis: The quest for mechanisms. The Journal of Clinical Investigation. 2013;**123**(5):1919-1921

[47] Galea GL, Meakin LB, Sugiyama T, Zebda N, Sunters A, Taipaleenmaki H, Stein GS, van Wijnen AJ, Lanyon LE, Price JS. Estrogen receptor alpha mediates proliferation of osteoblastic cells stimulated by estrogen and mechanical strain, but their acute down-regulation of the Wnt antagonist Sost is mediated by estrogen receptor beta. The Journal of Biological Chemistry. 2013;**288**(13):9035-9048

[48] Hong L, Zhang G, Sultana H, Yu Y, Wei Z. The effects of 17-beta estradiol on enhancing proliferation of human bone marrow mesenchymal stromal cells in vitro. Stem Cells and Development. 2011;**20**(5):925-931

[49] Hamada H, Kim MK, Iwakura A, Ii M, Thorne T, Qin G, Asai J, Tsutsumi Y, Sekiguchi H, Silver M, Wecker A, Bord E, Zhu Y, Kishore R, Losordo DW. Estrogen receptors alpha and beta mediate contribution of bone marrow-derived endothelial progenitor cells to functional recovery after myocardial infarction. Circulation. 2006;**114**(21):2261-2270

[50] Torres-Estay V, Carreño DV, San Francisco IF, Sotomayor P, Godoy AS, Smith GJ. Androgen receptor in human endothelial cells. Journal of Endocrinology. 2015;**224**(3):R131-R1R7

[51] Childs BG, Durik M, Baker DJ, van Deursen JM. Cellular senescence in aging and age-related disease: From mechanisms to therapy. Nature Medicine. 2015;**21**(12):1424-1435

[52] Bielak-Zmijewska A, Mosieniak G, Sikora E. Is DNA damage indispensable for stress-induced senescence? Mechanisms of Ageing and Development. March 2018;**170**:13-21

[53] Ito Y, Hoare M, Narita M. Spatial and temporal control of senescence. Trends in Cell Biology. 2017;**27**(11):820-832

[54] Rao SG, Jackson JG. SASP: Tumor suppressor or promoter? Yes! Trends in Cancer. 2016; **2**(11):676-687

[55] Byun HO, Lee YK, Kim JM, Yoon G. From cell senescence to age-related diseases: Differential mechanisms of action of senescence-associated secretory phenotypes. BMB Reports. 2015;**48**(10):549-558

[56] Davalli P, Mitic T, Caporali A, Lauriola A, D'Arca D. ROS, cell senescence, and novel molecular mechanisms in aging and age-related diseases. Oxidative Medicine and Cellular Longevity. 2016;**2016**:3565127

[57] Galluzzi L, Maiuri MC, Vitale I, Zischka H, Castedo M, Zitvogel L, Kroemer G. Cell death modalities: Classification and pathophysiological implications. Cell Death and Differentiation. 2007;**14**(7):1237-1243

[58] Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, Blagosklonny MV, El-Deiry WS, Golstein P, Green DR, Hengartner M, Knight RA, Kumar S, Lipton SA, Malorni W, Nunez G, Peter ME, Tschopp J, Yuan J, Piacentini M, Zhivotovsky B, Melino G. Classification of cell death: Recommendations of the nomenclature committee on cell death 2009. Cell Death and Differentiation. 2009;**16**(1):3-11

[59] Portal-Nunez S, Esbrit P, Alcaraz MJ, Largo R. Oxidative stress, autophagy, epigenetic changes and regulation by miRNAs as potential therapeutic targets in osteoarthritis. Biochemical Pharmacology. 2016;**108**:1-10

[60] Kroemer G, Levine B. Autophagic cell death: The story of a misnomer. Nature Reviews Molecular Cell Biology. 2008;**9**(12):1004-1010

[61] Vasconsuelo A, Pronsato L, Ronda AC, Boland R, Milanesi L. Role of 17beta-estradiol and testosterone in apoptosis. Steroids. 2011;**76**(12):1223-1231

[62] Maselli A, Matarrese P, Straface E, Canu S, Franconi F, Malorni W. Cell sex: A new look at cell fate studies. The FASEB Journal. 2009;**23**(4):978-984

[63] Pronsato L, Ronda A, Milanesi L, Vasconsuelo A, Boland R. Protective role of 17β-estradiol and testosterone in apoptosis of skeletal muscle. Actualizaciones en Osteologia. 2010;**6**(2):65-80

[64] Nilsen J, Chen S, Irwin RW, Iwamoto S, Brinton RD. Estrogen protects neuronal cells from amyloid beta-induced apoptosis via regulation of mitochondrial proteins and function. BMC Neuroscience. 2006;**7**:74

[65] Pike CJ, Nguyen TV, Ramsden M, Yao M, Murphy MP, Rosario ER. Androgen cell signaling pathways involved in neuroprotective actions. Hormones and Behaviour. 2008;**53**(5):693-705

[66] Palomar-Morales M, Morimoto S, Mendoza-Rodriguez CA, Cerbon MA. The protective effect of testosterone on streptozotocin-induced apoptosis in beta cells is sex specific. Pancreas. 2010;**39**(2):193-200

[67] Ackermann S, Hiller S, Osswald H, Losle M, Grenz A, Hambrock A. 17beta-estradiol modulates apoptosis in pancreatic beta-cells by specific involvement of the sulfonylurea receptor (SUR) isoform SUR1. The Journal of Biological Chemistry. 2009;**284**(8):4905-4913

[68] Ling S, Zhou L, Li H, Dai A, Liu JP, Komesaroff PA, Sudhir K. Effects of 17beta-estradiol on growth and apoptosis in human vascular endothelial cells: Influence of mechanical strain and tumor necrosis factor-alpha. Steroids. 2006;**71**(9):799-808

[69] Lu A, Frink M, Choudhry MA, Hubbard WJ, Loring W, Rue I, Bland KI, Chaudry IH. Mitochondria play an important role in 17β-estradiol attenuation of H_2O_2-induced rat endothelial cell apoptosis. American Journal of Physiology—Endocrinology and Metabolism. 2007;**292**(2):E585-EE93

[70] Seli E, Guzeloglu-Kayisli O, Cakmak H, Kayisli UA, Selam B, Arici A. Estradiol increases apoptosis in human coronary artery endothelial cells by up-regulating Fas and Fas ligand expression. The Journal of Clinical Endocrinology & Metabolism. 2006;**91**(12):4995-5001

[71] Ling S, Dai A, Williams MR, Myles K, Dilley RJ, Komesaroff PA, Sudhir K. Testosterone (T) enhances apoptosis-related damage in human vascular endothelial cells. Endocrinology. 2002;**143**(3):1119-1125

[72] Alvarez RJ, Gips SJ, Moldovan N, Wilhide CC, Milliken EE, Hoang AT, Hruban RH, Silverman HS, Dang CV, Goldschmidt-Clermont PJ. 17beta-estradiol inhibits apoptosis of endothelial cells. Biochemical and Biophysical Research Communications. 1997;**237**(2): 372-381

[73] Florian M, Magder S. Estrogen decreases TNF-alpha and oxidized LDL induced apoptosis in endothelial cells. Steroids. 2008;**73**(1):47-58

[74] Yang YC, Fu HC, Wu CY, Wei KT, Huang KE, Kang HY. Androgen receptor accelerates premature senescence of human dermal papilla cells in association with DNA damage. PLoS One. 2013;**8**(11):e79434

[75] Winiarska A, Mandt N, Kamp H, Hossini A, Seltmann H, Zouboulis CC, Blume-Peytavi U. Effect of 5alpha-dihydrotestosterone and testosterone on apoptosis in human dermal papilla cells. Skin Pharmacology and Physiology. 2006;**19**(6):311-321

[76] Verzola D, Villaggio B, Procopio V, Gandolfo MT, Gianiorio F, Fama A, Tosetti F, Traverso P, Deferrari G, Garibotto G. Androgen-mediated apoptosis of kidney tubule cells: Role of c-Jun amino terminal kinase. Biochemical and Biophysical Research Communications. 2009;**387**(3):531-536

[77] Gu S, Papadopoulou N, Gehring E-M, Nasir O, Dimas K, Bhavsar SK, Föller M, Alevizopoulos K, Lang F, Stournaras C. Functional membrane androgen receptors in colon tumors trigger pro-apoptotic responses in vitro and reduce drastically tumor incidence in vivo. Molecular Cancer. 2009;**8**:114

[78] Jin L, Ai X, Liu L, Wang Z, Cheng Y, Qiao Z. Testosterone induces apoptosis via Fas/FasL-dependent pathway in bone marrow-derived macrophages. Methods and Findings in Experimental and Clinical Pharmacology. 2006;**28**(5):283-293

[79] Penaloza C, Estevez B, Orlanski S, Sikorska M, Walker R, Smith C, Smith B, Lockshin RA, Zakeri Z. Sex of the cell dictates its response: Differential gene expression and sensitivity to cell death inducing stress in male and female cells. FASEB Journal. 2009;**23**(6):1869-1879

[80] Straface E, Gambardella L, Brandani M, Malorni W. Sex differences at cellular level: "Cells have a sex". In: Regitz-Zagrosek V, editor. Sex and Gender Differences in Pharmacology. Berlin, Heidelberg: Springer Berlin Heidelberg; 2012. pp. 49-65

[81] Straface E, Vona R, Gambardella L, Ascione B, Marino M, Bulzomi P, Canu S, Coinu R, Rosano G, Malorni W, Franconi F. Cell sex determines anoikis resistance in vascular smooth muscle cells. FEBS Letters. 2009;**583**(21):3448-3454

[82] Du L, Hickey RW, Bayir H, Watkins SC, Tyurin VA, Guo F, Kochanek PM, Jenkins LW, Ren J, Gibson G, Chu CT, Kagan VE, Clark RSB. Starving neurons show sex difference in autophagy. Journal of Biological Chemistry. 2009;**284**(4):2383-2396

[83] Campesi I, Straface E, Occhioni S, Montella A, Franconi F. Protein oxidation seems to be linked to constitutive autophagy: A sex study. Life Sciences. 2013;**93**(4):145-152

[84] Camuzard O, Santucci-Darmanin S, Breuil V, Cros C, Gritsaenko T, Pagnotta S, Cailleteau L, Battaglia S, Panaia-Ferrari P, Heymann D, Carle GF, Pierrefite-Carle V. Sex-specific autophagy modulation in osteoblastic lineage: A critical function to counteract bone loss in female. Oncotarget. 2016;**7**(41):66416-66428

[85] Lee WJ, Lee SC, Lee JH, Rho GJ, Lee SL. Differential regulation of senescence and in vitro differentiation by 17beta-estradiol between mesenchymal stem cells derived from male and female mini-pigs. Journal of Veterinary Science. 2016;**17**(2):159-170

[86] Ortona E, Matarrese P, Malorni W. Taking into account the gender issue in cell death studies. Cell Death & Disease. 2014;**5**:e1121

[87] Malorni W, Straface E, Matarrese P, Ascione B, Coinu R, Canu S, Galluzzo P, Marino M, Franconi F. Redox state and gender differences in vascular smooth muscle cells. FEBS Letters. 2008;**582**(5):635-642

[88] Satoh M, Matter CM, Ogita H, Takeshita K, Wang C-Y, Dorn GW, Liao JK. Inhibition of apoptosis-regulated signaling Kinase-1 and prevention of congestive heart failure by estrogen. Circulation. 2007;**115**(25):3197-3204

[89] Liu H, Yanamandala M, Lee TC, Kim JK. Mitochondrial p38beta and manganese superoxide dismutase interaction mediated by estrogen in cardiomyocytes. PLoS One. 2014; **9**(1):e85272

[90] Strehlow K, Rotter S, Wassmann S, Adam O, Grohé C, Laufs K, Böhm M, Nickenig G. Modulation of antioxidant enzyme expression and function by estrogen. Circulation Research. 2003;**93**(2):170

[91] McCullough LD, Zeng Z, Blizzard KK, Debchoudhury I, Hurn PD. Ischemic nitric oxide and poly (ADP-ribose) polymerase-1 in cerebral ischemia: Male toxicity, female protection. Journal of Cerebral Blood Flow and Metabolism. 2005;**25**(4):502-512

[92] Liu F, Li Z, Li J, Siegel C, Yuan R, McCullough LD. Sex differences in caspase activation after stroke. Stroke. 2009;**40**(5):1842-1848

[93] Benjamin EJ, Blaha MJ, Chiuve SE, Cushman M, Das SR, Deo R, de Ferranti SD, Floyd J, Fornage M, Gillespie C, Isasi CR, Jimenez MC, Jordan LC, Judd SE, Lackland D, Lichtman JH, Lisabeth L, Liu S, Longenecker CT, Mackey RH, Matsushita K, Mozaffarian D, Mussolino ME, Nasir K, Neumar RW, Palaniappan L, Pandey DK, Thiagarajan RR,

Reeves MJ, Ritchey M, Rodriguez CJ, Roth GA, Rosamond WD, Sasson C, Towfighi A, Tsao CW, Turner MB, Virani SS, Voeks JH, Willey JZ, Wilkins JT, Wu JH, Alger HM, Wong SS, Muntner P. Heart disease and stroke Statistics-2017 update: A report from the American Heart Association. Circulation. 2017;**135**(10):e146-e603

[94] Koellhoffer EC, McCullough LD. The effects of estrogen in ischemic stroke. Translational Stroke Research. 2013;**4**(4):390-401

[95] Jog NR, Caricchio R. Differential regulation of cell death programs in males and females by poly (ADP-ribose) polymerase-1 and 17beta estradiol. Cell Death & Disease. 2013;**4**:e758

[96] Klinge CM. Estrogens regulate life and death in mitochondria. Journal of Bioenergetics and Biomembranes. 2017;**49**(4):307-324

[97] Sanchez MI, Shearwood AM, Chia T, Davies SM, Rackham O, Filipovska A. Estrogen-mediated regulation of mitochondrial gene expression. Molecular Endocrinology. 2015; **29**(1):14-27

[98] Nilsen J, Irwin RW, Gallaher TK, Brinton RD. Estradiol in vivo regulation of brain mitochondrial proteome. The Journal of Neuroscience. 2007;**27**(51):14069

[99] Razmara A, Duckles SP, Krause DN, Procaccio V. Estrogen suppresses brain mitochondrial oxidative stress in female and male rats. Brain Research. 2007;**1176**:71-81

[100] Stirone C, Duckles SP, Krause DN, Procaccio V. Estrogen increases mitochondrial efficiency and reduces oxidative stress in cerebral blood vessels. Molecular Pharmacology. 2005;**68**(4):959

[101] Velarde MC. Mitochondrial and sex steroid hormone crosstalk during aging. Longevity & Healthspan. 2014;**3**(1):2

[102] Salmon AB, Richardson A, Perez VI. Update on the oxidative stress theory of aging: Does oxidative stress play a role in aging or healthy aging? Free Radical Biology & Medicine. 2010;**48**(5):642-655

[103] Haigis MC, Yankner BA. The aging stress response. Molecular Cell. 2010;**40**(2):333-344

[104] Tower J. Sex-specific gene expression and life span regulation. Trends in Endocrinology and Metabolism. 2017;**28**(10):735-747

[105] Tower J. Mitochondrial maintenance failure in aging and role of sexual dimorphism. Archives of Biochemistry and Biophysics. 2015;**576**:17-31

[106] Park JH, Zhuang J, Li J, Hwang PM. p53 as guardian of the mitochondrial genome. FEBS Letters. 2016;**590**(7):924-934

[107] Kim S, Koh H. Role of FOXO transcription factors in crosstalk between mitochondria and the nucleus. Journal of Bioenergetics and Biomembranes. 2017;**49**(4):335-341

[108] Oertelt-Prigione S, Regitz-Zagrosek V. Sex and Gender Aspects in Clinical Medicine. Springer-Verlag London Ltd: London, UK; 2012

[109] Schenck-Gustafsson K, DeCola PR, Pfaff DW, Pisetsky DS. Handbook of Clinical Gender Medicine. Ergolding, Germany. Basel (CH): Karger Medical and Scientific Publishers; 2012

[110] Legato MJ. Principles of Gender-Specific Medicine: Gender in the Genomic Era. Chennai, India: Elsevier Inc, Academic Press; 2017

[111] Kramer A, Pippias M, Stel VS, Bonthuis M, Abad Diez JM, Afentakis N, Alonso de la Torre R, Ambuhl P, Bikbov B, Bouzas Caamaño E, Bubic I, Buturovic-Ponikvar J, Caskey FJ, Castro de la Nuez P, Cernevskis H, Collart F, Comas Farnés J, Garcia Bazaga MdlÁ, De Meester J, Ferrer Alamar M, Finne P, Garneata L, Golan E, Heaf JG, Hemmelder M, Ioannou K, Kantaria N, Kolesnyk M, Kramar R, Lassalle M, Lezaic V, Lopot F, Macário F, Magaz A, Martín-Escobar E, Metcalfe W, Ots-Rosenberg M, Palsson R, Piñera Celestino C, Resić H, Rutkowski B, Santiuste de Pablos C, Spustová V, Stendahl M, Strakosha A, Süleymanlar G, Torres Guinea M, Varberg Reisæter A, Vazelov E, Ziginskiene E, Massy ZA, Wanner C, Jager KJ, Noordzij M. Renal replacement therapy in Europe: A summary of the 2013 ERA-EDTA Registry Annual Report with a focus on diabetes mellitus. Clinical Kidney Journal. 2016;**9**(3):457-469

[112] Saran R, Robinson B, Abbott KC, Agodoa LY, Ayanian J, Bragg-Gresham J, Balkrishnan R, Chen JL, Cope E, Eggers PW, Gillen D, Gipson D, Hailpern SM, Hall YN, Han Y, He K, Herman W, Heung M, Hutton D, Jacobsen SJ, Kalantar-Zadeh K, Kovesdy CP, Li Y, Lu Y, Molnar MZ, Morgenstern H, Nallamothu B, Nguyen DV, O'Hare AM, Obi Y, Plattner B, Pisoni R, Port FK, Rao P, Ravel V, Rhee CM, Sakhuja A, Schaubel DE, Selewski DT, Sim JJ, Song P, Streja E, Kurella Tamura M, Tentori F, White S, Woodside K, Hirth RA, Shahinian VUS. Renal data system 2016 annual data report: Epidemiology of kidney disease in the United States. American Journal of Kidney Disease. 2017;**69**(3S1):A7-A8

[113] USRDS. United States Renal Data System 2016. USRDS annual data report: Epidemiology of kidney disease in the United States. National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD, 2016 http://www.usrds.org. 2016

[114] Kramer A, Stel V, Zoccali C, Heaf J, Ansell D, Grönhagen-Riska C, Leivestad T, Simpson K, Pálsson R, Postorino M, Jager K. An update on renal replacement therapy in Europe: ERA–EDTA registry data from 1997 to 2006. Nephrology Dialysis Transplantation. 2009; **24**(12):3557-3566

[115] Turin TC, Tonelli M, Manns BJ, Ahmed SB, Ravani P, James M, Hemmelgarn BR. Lifetime risk of ESRD. Journal of the American Society of Nephrology. 2012;**23**(9):1569-1578

[116] Kiberd BA, Clase CM. Cumulative risk for developing end-stage renal disease in the US population. Journal of the American Society of Nephrology. 2002;**13**(6):1635-1644

[117] Neugarten J, Golestaneh L. Gender and the prevalence and progression of renal disease. Advances in Chronic Kidney Disease. 2013;**20**(5):390-395

[118] Carrero JJ. Gender differences in chronic kidney disease: Underpinnings and therapeutic implications. Kidney and Blood Pressure Research. 2010;**33**(5):383-392

[119] Kummer S, von Gersdorff G, Kemper M, Oh J. The influence of gender and sexual hormones on incidence and outcome of chronic kidney disease. Pediatric Nephrology. 2012;**27**(8):1213-1219

[120] Seliger SL, Davis C, Stehman-Breen C. Gender and the progression of renal disease. Current Opinion in Nephrology and Hypertension. 2001;**10**(2):219-225

[121] Kang D-H, Yu ES, Yoon K-I, Johnson R. The impact of gender on progression of renal disease: Potential role of estrogen-mediated vascular endothelial growth factor regulation and vascular protection. American Journal of Pathololgy. 2004;**164**(2):679-688

[122] Iliescu R, Reckelhoff JF. Sex and the kidney. Hypertension. 2008;**51**(4):1000-1001

[123] Maric C. Sex differences in cardiovascular disease and hypertension: Involvement of the renin-angiotensin system. Hypertension. 2005;**46**(3):475-476

[124] Kwan G, Neugarten J, Sherman M, Ding Q, Fotadar U, Lei J, Silbiger S. Effects of sex hormones on mesangial cell proliferation and collagen synthesis. Kidney International. 1996;**50**(4):1173-1179

[125] Oberley TD, Lauchner LJ, Pugh TD, Gonzalez A, Goldfarb S, Li SA, Li JJ. Specific estrogen-induced cell proliferation of cultured Syrian hamster renal proximal tubular cells in serum-free chemically defined media. Proceedings of the National Academy of Sciences. 1989;**86**(6):2107-2111

[126] Han H, Lim M, Lee Y, Kim E, Jeon Y, Lee J. Effects of TCDD and estradiol-17beta on the proliferation and Na+/glucose cotransporter in renal proximal tubule cells. Toxicology In Vitro. 2005;**19**(1):21-30

[127] Seppi T, Prajczer S, Dorler MM, Eiter O, Hekl D, Nevinny-Stickel M, Skvortsova I, Gstraunthaler G, Lukas P, Lechner J. Sex differences in renal proximal tubular cell homeostasis. Journal of the American Society of Nephrology. 2016;**27**(10):3051-3062

[128] Lechner J, Malloth NA, Jennings P, Hekl D, Pfaller W, Seppi T. Opposing roles of EGF in IFN-{alpha}-induced epithelial barrier destabilization and tissue repair. American Journal of Physiology—Cell Physiology. 2007;**293**(6):C1843-C1850

[129] Lechner J, Malloth N, Seppi T, Beer B, Jennings P, Pfaller W. IFN-{alpha} induces barrier destabilization and apoptosis in renal proximal tubular epithelium. American Journal of Physiology—Cell Physiology. 2008;**294**(1):C153-CC60

Section 3

Integrated Systems

Chapter 4

Gateway Reflex: A Neuro-Immune Crosstalk for Organ-Specific Disease Development

Daisuke Kamimura, Yuki Tanaka, Takuto Ohki and Masaaki Murakami

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.77112

Abstract

Homeostasis of the central nervous system (CNS) is strictly regulated by a unique structure of blood vessels, the blood-brain barrier (BBB). Experimental and clinical evidence has revealed that abnormalities in the BBB in chronic inflammatory diseases such as multiple sclerosis (MS). By using an animal model of MS, we identified novel neuro-immune crosstalk to explain how pathogenic immune cells enter the CNS to disrupt its homeostasis, a phenomenon we named the gateway reflex. Regional neural inputs such as gravity, electricity, pain or chronic stress cause specific neural activation to create a gateway of immune cells, particularly pathogenic ones, at specific blood vessels. Moreover, the recently discovered stress-induced gateway reflex uncovered a stress-induced neural link between the brain, gastrointestine, and heart. Thus, the gateway reflex is critical for the homeostasis of various organs, and aberrant activation of neural pathways by the gateway reflex disrupts normal organ homeostasis. The inflammatory reflex is another mechanism for local neuro-immune interactions. It potently exerts a cholinergic anti-inflammatory effect on various disease conditions. In this section, we discuss emerging roles for local neuro-immune interactions, with a special focus on the gateway reflex.

Keywords: gateway reflex, experimental autoimmune encephalomyelitis, central nervous system, chemokines, pathogenic CD4+ T cells

1. Introduction

A variety of environmental stimulations such as light, temperature, sound, and so on activates specific neurons to trigger biological responses. Gravity is another stimulus on land animals. Without sufficient gravity stimulation, physical functions including bone mass and

muscle strength change, as observed in astronauts who stayed in the International Space Station [1, 2]. In addition to these physical stimulations, various events in daily social interactions associated with psychological alterations including anxiety, mental stresses or positive emotions are also stimulatory factors that trigger specific neural pathways to affect body functions. A well-studied mechanism to cope with these stimulations includes the release of glucocorticoids via the hypothalamus-pituitary-adrenal grand (HPA) axis, which systemically changes various physiological functions including the immune system [3, 4]. Besides this systemic regulation, we and other groups have identified local regulations of the inflammatory status by various environmental cues that cause specific neural activations. Here we summarize examples of specific neuro-immune interactions in organ homeostasis.

2. Main text

2.1. Gravity gateway reflex

The central nervous system (CNS) is considered an immune privileged site due to structural protections by the blood-brain barrier (BBB) [5]. The BBB is formed by tight cell-cell interactions between blood endothelial cells and tight liner sheets, a structure that lies outside the basement membrane and consists of pericytes, neurons, microglias, and astrocytes. Tight junctions by the interactions of tight-junction molecules including claudins and occludins are critical for the cell-cell interactions in the BBB that sequester cerebrospinal fluid from circulating blood components [6]. However, despite the BBB, there exist a certain number of immune cells in the CNS to prevent brain tumors and viral infections. In pathological conditions of the CNS, excessive immune cells from the blood accumulate from the breached BBB to cause chronic inflammatory diseases including multiple sclerosis (MS). It is also known that brain micro-inflammation is associated with neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease [7]. Inflammatory cytokines including IL-1β, IL-17A, IFNγ and TNFα increase the BBB permeability, while chemokines recruit immune cells from the blood to cause inflammation [8–10]. However, where and how local CNS inflammation is induced in the BBB is poorly understood.

MS is a chronic inflammatory disease of the CNS. It is characterized by impairments in sensory, motor, autonomic, and neurocognitive functions due to autoimmune-mediated demyelination in CNS nerves [11]. Genetic factors strongly contribute to the MS pathogenesis, and genome-wide association studies (GWAS) have revealed that certain alleles of major histocompatibility complex (MHC) class II genes including HLA-DRB1*15:01 and HLA-DRB1*13:03 and genes involved in CD4+ T cell activation and homeostasis such as IL-2Rα and IL-7R are genetically associated with MS development [12–15]. These genetic results strongly suggest that autoreactive CD4+ T cells play a vital role in the MS pathogenesis and are supported by animal models of MS including experimental autoimmune encephalomyelitis (EAE) [16–19]. Using an adaptive transfer model of EAE [20], we investigated how and where myelin-autoreactive pathogenic CD4+ T cells initially invade the CNS. Whole-mount sections of adult mice with pathogenic CD4+ T cell transfer sacrificed just before the onset of

http://dx.doi.org/10.5772/intechopen.77112

EAE symptoms revealed that pathogenic CD4+ T cells mainly accumulated at the L5 spinal cord, around the dorsal vessels in particular [18]. On the other hand, no accumulation was observed in the brain or upper spinal levels at the preclinical time point of EAE. Various chemokines including CCL20 attract type-17 CD4+ T (Th17) cells, which are known to have a key pathogenic role in EAE [21, 22], to accumulate more in the dorsal vessels of L5 than L1 cord. Indeed, CCL20 neutralization or deficiency of its receptor, CCR6, on pathogenic CD4+ T cells abrogated the cell accumulation in the L5 spinal cord [18].

Interestingly, even without EAE induction, chemokine levels were higher in the L5 dorsal vessels than in the L1 cord. These results suggest a distinct property of L5 dorsal vessels both in the presence and absence of pathogenic CD4+ T cells. The L5 spinal level has the largest dorsal root ganglion (DRG) in both human and mice, and it is known that sensory neurons in the L5 DRG distribute to the soleus muscles, the main anti-gravity muscles of the body [23, 24]. These facts led us to hypothesize a link between gravity, L5 vessels and local inflammation. We examined this possibility using a ground experiment employed by the National Aeronautics and Space Administration (NASA) and Japan Aerospace Exploration Agency (JAXA) [25, 26]. The tail suspension method puts mice in a handstand position, such that the hind limbs are released from gravity stimulation. As hypothesized, after the tail suspension, chemokine expressions of the L5 dorsal vessels were reduced and pathogenic CD4+ T cells hardly invaded the L5 cord. Chemokines were instead upregulated at the cervical cords as though another gateway for immune cells was formed by the greater gravity stimulation on the arm muscles imposed by the tail suspension [18]. Consistently, the tail suspension significantly reduced the expression of a neural activation marker, c-Fos, in the L5 DRG, suggesting a correlation between regional neural activation and local inflammation in the CNS. Consistently, stimulation of the soleus muscles by weak electric pulses, which mimic gravity-mediated sensory activation, during tail suspension restored chemokine expression, pathogenic CD4+ T cell accumulation, and c-Fos levels at the L5 dorsal vessels [18]. That study identified L5 dorsal vessels as a blood vessel gateway for immune cells including pathogenic CD4+ T cells to the CNS, and revealed that regional sensory neural activation by gravity affects CNS homeostasis and causes local inflammation at L5 dorsal blood vessels when CNS-autoreactive pathogenic CD4+ T cells are present. This phenomenon, which represents a novel neuro-immune interaction, was termed the "gateway reflex" [27–34]. Since then, we have found various types of gateway reflexes depending on the neural stimulation. Thus, the abovementioned example is known as the gravity gateway reflex (**Figure 1**).

How the regional neural pathway by gravity stimulation regulates chemokine expressions in L5 blood vessels is an important question. Contribution of the autonomic nervous system was suggested. In fact, neural activation examined by c-Fos expression was higher in sympathetic ganglions at the L5 cord than at the L1 cord. In addition, the blood flow speed of the L5 dorsal vessels but not of other blood vessels including the L1 dorsal vessels, femoral artery or portal vein, slowed after tail suspension. Functionally, the treatment of mice with β-adrenergic receptor antagonists or chemical sympathectomy inhibited chemokine expressions, the accumulation of pathogenic CD4+ T cells at the L5 dorsal vessels, and the clinical scores of EAE [18]. Norepinephrine is one of the major neurotransmitters of sympathetic nerves. Indeed, in vitro experiments suggest that norepinephrine enhances chemokine expressions from

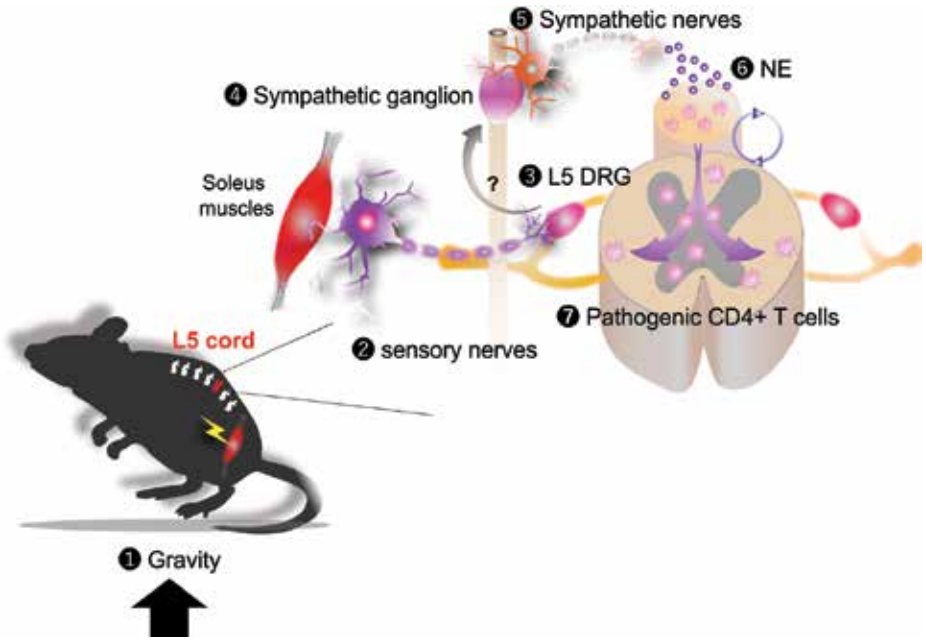

Figure 1. Gravity gateway reflex. Stimulation of the soleus muscles by gravity (1) induces the activation of specific sensory nerves (2). The cell bodies of these sensory neurons are located at the dorsal root ganglion (DRG) of the fifth lumbar (L5) spinal cord (3). Neural activation via the L5 DRG neurons travels to the L5 sympathetic ganglion (4) and induces the activation of sympathetic nerves (5), which results in norepinephrine (NE) secretion (6) at the L5 dorsal vessels. NE enhances the inflammation amplifier in the L5 dorsal vessels, causing an upregulation of chemokines and recruiting pathogenic CD4+ T cells from the vessels (7).

endothelial cell lines with activation of the inflammation amplifier [18]. The inflammation amplifier is a molecular mechanism that operates in nonimmune cells including endothelial cells to produce a large amount of pro-inflammatory mediators including chemokines, cytokines and growth factors upon the concomitant activation of two transcription factors, NF-κB and STAT3 [35–37]. The co-activation of NF-κB and STAT3 by cytokines such as IL-17, IL-6, and TNFα augments NF-κB activity and upregulates NF-κB-target genes including chemokines, which can be further enhanced by neurotransmitters including norepinephrine and ATP (**Figure 2**) [18, 38]. The contribution of nonimmune cells to chronic inflammation via the inflammation amplifier has been found significant in the development of various disease models and evidence of inflammation amplifier activation has been observed in patients with MS, rheumatoid arthritis, atherosclerosis, and chronic rejection after lung transplantation [39–45]. Thus, the gateway reflex represents novel local neuro-immune communication involving the activation of specific sensory and sympathetic neurons for the formation of blood vessel gateways (**Figure 1**). Because chemokine levels are constantly higher at the L5 dorsal vessels under normal conditions without EAE induction, the gateway reflex could have a physiological role as well. Indeed, it is reported in mice that after a learning task, CD4+ T cells, but not CD8+ T cells, accumulated in the CNS, and mice devoid of CD4+ T cells showed impaired learning performance and neurogenesis in the hippocampus [46–48]. The gravity gateway reflex may be utilized by these cells to enter the CNS to maintain and/or control the homeostasis and function of the CNS.

http://dx.doi.org/10.5772/intechopen.77112

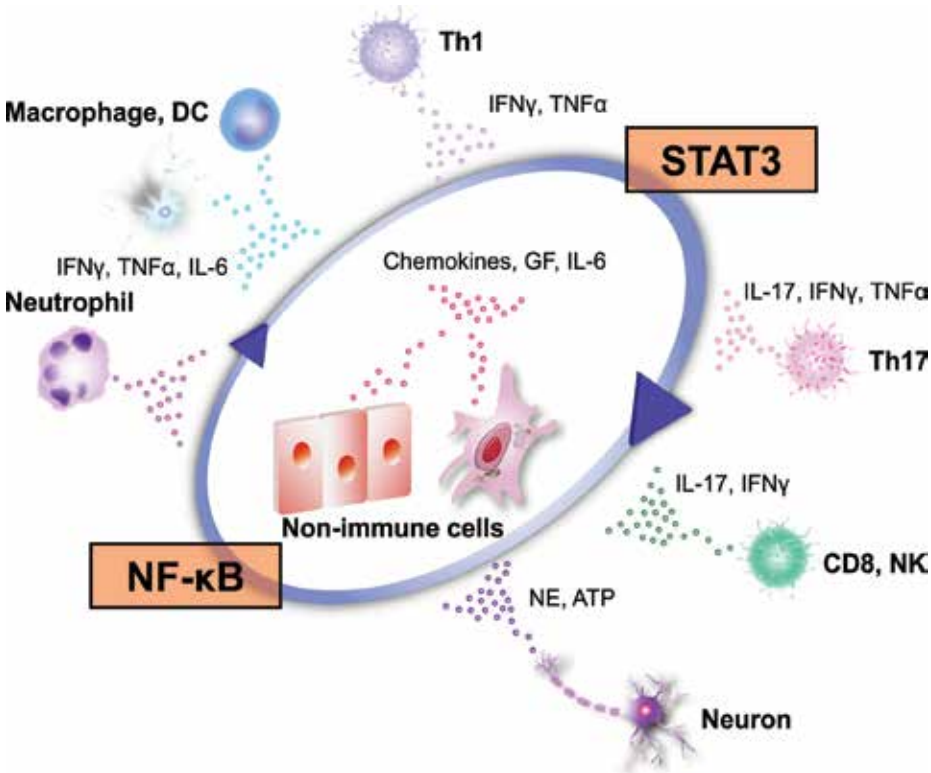

Figure 2. Inflammation amplifier. Co-activation of the transcription factors NF-κB and STAT3 in nonimmune cells such as endothelial cells and fibroblasts induces a synergistic effect on the production of inflammation factors such as chemokines, growth factors, and IL-6. Various factors activating NF-κB and STAT3 including IL-17, TNFα, and IL-6 can drive the amplifier. Secreted IL-6 is thought to act on nonimmune cells to form a positive feedback loop for this synergistic effect. Massive production of chemokines and growth factors by the inflammation amplifier play an essential role in the pathogenesis of many inflammatory diseases. DC, dendritic cells; GF, growth factors; NK, natural killer cells; NE, norepinephrine; Th, helper T cells.

2.2. Electric gateway reflex

The gateway reflex is not specific to the soleus-L5 axis because electrical stimulation of regional sensory neurons in different muscles can induce the formation of gateways in the dorsal vessels of different spinal cord levels. For instance, electric stimulation of the quadriceps, which are controlled by the L3 DRG neurons, induces chemokine expressions at the L3 dorsal vessels. Similarly, electric stimulations of the triceps upregulate chemokines at the dorsal vessels of the cervical to thoracic spinal cords (**Figure 3**) [18]. These results suggest that the electric gateway reflex can be artificially controlled, raising a possibility for a therapeutic application of the gateway reflex to various CNS diseases like MS and brain tumors.

2.3. Pain gateway reflex

We have examined whether other sensory stimulations can also generate the gateway reflex. We focused on pain because it is a tonic sensory stimulation [49, 50] and associated with various diseases that significantly compromise quality of life [51]. Some studies suggest that

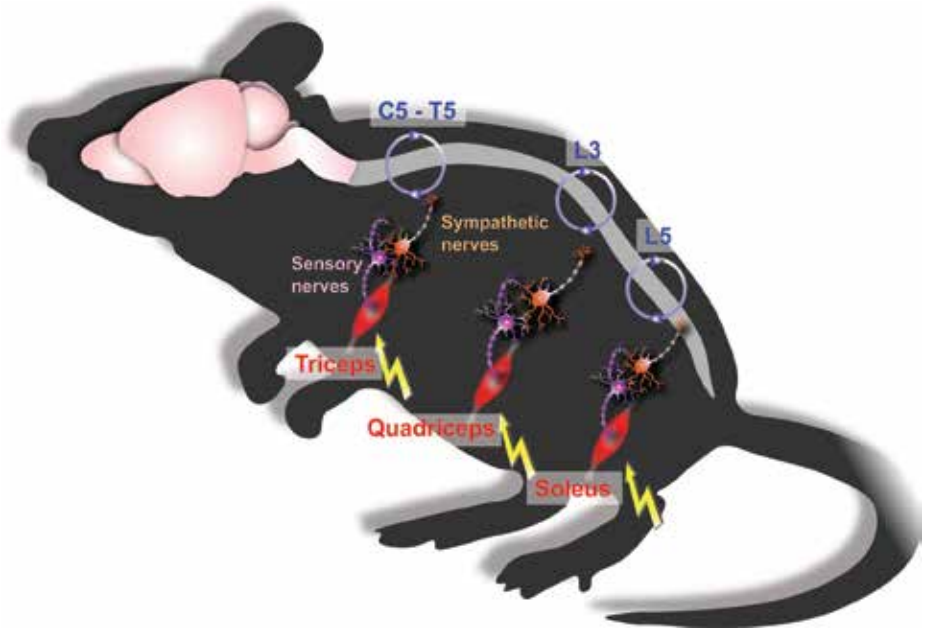

Figure 3. Electric gateway reflex. Electric stimulation to the triceps induces chemokine upregulation at the dorsal vessels of the fifth cervical (C5) to fifth thoracic (T5) spinal cord through sensory-sympathetic neural activation, which leads to activation of the inflammation amplifier at the C5-T5 dorsal vessels. In a similar fashion, electric stimulation of the quadriceps and soleus muscles induces a gateway at the L3 and L5 dorsal vessels, respectively.

pain occurrence is positively correlated with disease severity in MS [52–54], and mechanical allodynia and thermal hyperalgesia are induced in mice during EAE [55, 56]. In the adoptive transfer EAE model we used, recipient mice that received myelin-specific pathogenic CD4+ T cells developed transient paralysis, but then recovered and hardly relapsed even 100 days later. To examine how pain affects EAE symptoms, we induced pain sensation in mice by partial ligation of the middle branch of the trigeminal nerves, which are composed of sensory nerves alone [57]. Pain induction with pathogenic CD4+ T cell transfer significantly worsened the paralysis. By contrast, pain medicines improved EAE development [19]. Thus, pain is not simply an alert of the disease or injury status, but it also has a pathogenic role, triggering EAE relapse. Since a large part of MS patients show relapse and remission and pain is more frequently claimed by MS patients with higher disease scores [52, 53], we examined the effects of pain induction in mice that had recovered from EAE (EAE-recovered mice). As expected, EAE-recovered mice showed a clear sign of relapse after the partial ligation of the trigeminal nerve or injection of pain-inducing chemicals such as substance P and capsaicin [19]. As described above, under normal conditions, the blood vessel gateway for immune cells is the L5 dorsal vessels due to the effect of gravity [18]. To identify specific blood vessels that act as a gateway for immune cells that cause the pain-induced relapse, immunohistological examination of the CNS from EAE-recovered mice was performed. Although the motility of EAE-recovered mice was not significantly different from normal healthy mice, the meningeal region of the L5 cord of EAE-recovered mice contained a high number of periphery-derived

monocytes that expressed high levels of MHC class II [18]. These MHC class II high monocytes distributed around the meningeal region of the L5 cord in EAE-recovered mice. After pain induction, however, these cells accumulated at the ventral vessels of the L5 cord bilaterally. Experimental evidence that (1) norepinephrine signaling could be detected around the L5 ventral vessels, (2) MHC class II high monocytes expressed chemokine receptor CX3CR1, and (3) MHC class II high monocytes secreted chemokine CX3CL1, a ligand for CX3CR1, after norepinephrine stimulation suggested an auto/paracrine loop is responsible for the accumulation of MHC class II high monocytes around the L5 ventral vessels via norepinephrine regulation of the CX3CL1-CX3CR1 axis. Because freshly isolated MHC class II high monocytes from EAE-recovered mice have autoantigen (MOG)-presenting capacity without additional peptide loading, it can be suggested that MHC class II high monocytes accumulated around the L5 ventral vessels would activate pathogenic CD4+ T cells through autoantigen presentation, followed by regional inflammation and disease relapse. Suppression of norepinephrine signaling by a β1 blocker or sympathetic nerve ablation by chemical sympathectomy inhibited the pain-mediated accumulation of MHC class II high monocytes around the L5 ventral vessels [19]. These results identified the L5 ventral vessels as the gateway for pain-induced relapse in the EAE model (**Figure 4**). This pain gateway reflex is the third example of the gateway reflex following the aforementioned gravity and electric examples.

2.4. Stress gateway reflex

Chronic stresses deteriorate illness, an effect attributed to the proverbs "Illness starts in the mind" and "Care killed the cat." Chronic stress conditions often cause gastric and intestinal diseases via the brain-gut axis. Although these diseases are well known and often experienced, the underlying molecular mechanisms remain to be elucidated. Stresses generate neural activations involving multiple brain areas such as the paraventricular nucleus (PVN), dorsomedial nucleus of hypothalamus (DMH), dorsal motor nucleus of the vagal nerve (DMX), and vagal nerve pathway [58]. We therefore hypothesized that chronic stresses might induce a specific gateway reflex. Indeed, experiments confirmed the stress gateway reflex. We serendipitously found that micro-inflammation induced by the stress gateway reflex activates an otherwise resting neural circuit to enhance a stress response that causes fatal gastrointestinal and heart failure in mice [38]. Therefore, the stress gateway reflex may explain the mechanisms for the abovementioned proverbs, as described below.

The gravity gateway reflex directs donor pathogenic CD4+ T cells to the dorsal vessels of the L5 spinal cord under normal conditions (**Figure 1**). However, under chronic stress conditions, the cells instead invade specific blood vessels of the boundary area of the third ventricle (3V), thalamus, and part of hippocampus [dentate gyrus, (DG)] to establish micro-inflammation. Therefore, chronic stresses can alter the location of the immune cell gateway from the L5 dorsal vessels to specific vessels in the brain. Immunohistochemistry of c-Fos revealed that the resulting micro-inflammation led to enhanced neural activations in the PVN and DMH, which are stress sensing areas of the hypothalamus. Neural tracing experiments identified direct connections via noradrenergic neurons between the PVN and the specific vessels of the boundary area of 3V, thalamus and DG. Moreover, these experiments showed direct

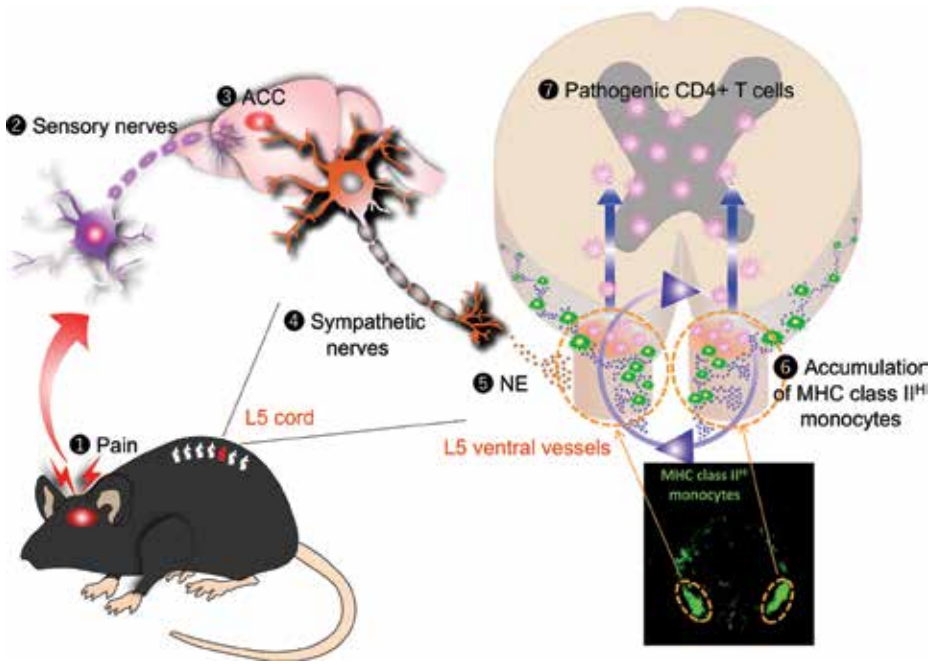

Figure 4. Pain gateway reflex. Pain induces nociceptive sensory nerve stimulation (1, 2), leading to activation of the anterior cingulate cortex (ACC), a pain-processing area of the brain (3). Specific sympathetic nerves are then activated (4) to induce norepinephrine (NE) release around the ventral vessels of the spinal cords (5). NE around the L5 ventral vessels induces the production of chemokine CX3CL1 from MHC class II high (MHC class II^{HI}) monocytes, further recruiting these cells in an auto/paracrine manner (6). MHC class II high monocytes are able to present myelin autoantigens to activate pathogenic CD4+ T cells, leading to disease relapse (7).

connections via nonnoradrenergic neurons between the specific vessels and the DMH. Since the PVN is a key orchestrator of stress signals, its activation is expected to affect specific blood vessels via a new noradrenergic neural pathway. Indeed, the expression of chemokines such as CCL5 was upregulated at specific vessels in mice with chronic stress alone. CNS-reactive pathogenic CD4+ T cells in the blood circulation of the stressed mice sensed the upregulation of CCL5, caused micro-inflammation with peripheral derived MHC class II monocytes at specific vessels of the boundary area of the 3V, thalamus, and DG [38]. ATP is released during an inflammatory response, but it also acts as a neurotransmitter [59, 60]. The injection of an ATP receptor antagonist at specific blood vessels of the boundary area of the 3V, thalamus and DG suppressed both neural activation in the DMH and the mortality rate of EAE mice with chronic stress. Importantly, the direct injection of ATP or cytokines at these specific vessels, which mimicked the micro-inflammation, induced fatal gastrointestinal dysfunction in stressed mice without EAE induction. These results suggest that brain micro-inflammation at specific vessels of the boundary area of the 3V, thalamus, and DG activates a resting neural pathway through ATP production, thus strongly enhancing the stress response to cause fatal gastrointestinal damage via the DMX and vagal nerve activation. These results uncovered the stress gateway reflex acting as a direct link between micro-inflammation at a particular site in the brain and gastrointestinal homeostasis (**Figure 5**) [38].

http://dx.doi.org/10.5772/intechopen.77112

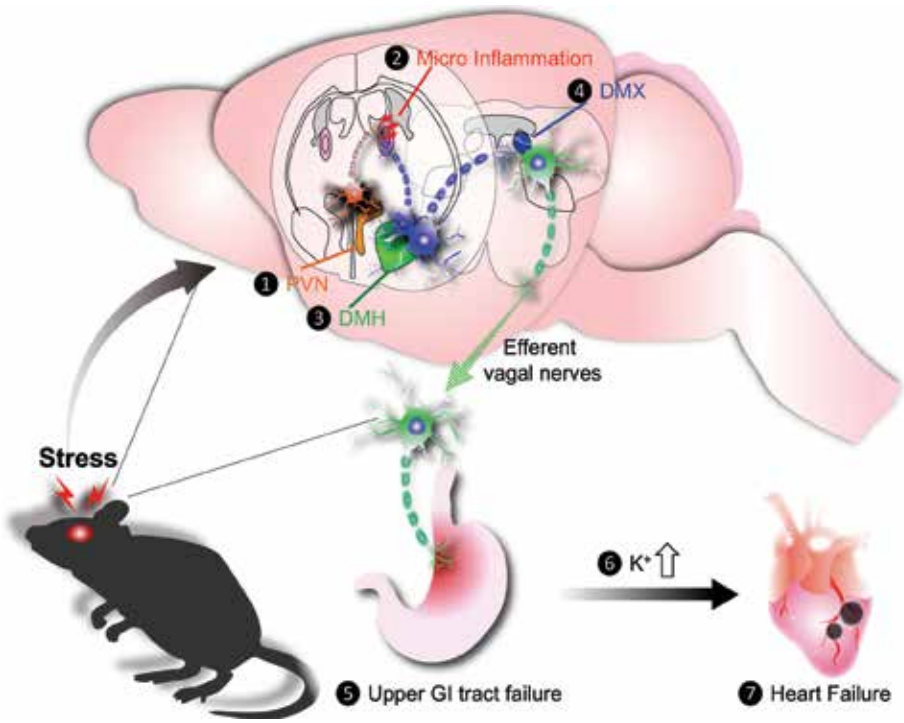

Figure 5. Stress gateway reflex. Chronic stress induces activation of the paraventricular nucleus (PVN) (1). We identified neural connections from the PVN to the specific vessels of the boundary area of the third ventricle, thalamus and dentate gyrus, which induces micro-inflammation around the specific vessels (2). The resulting micro-inflammation induces activation of a neural pathway that connects to the dorsomedial nucleus of hypothalamus (DMH) (3) and dorsal motor nucleus of the vagal nerve (DMX) (4), resulting in severe upper gastrointestinal (GI) tract failure via efferent vagal nerves (5). The increase of potassium ions (K^+) in blood circulation by the upper GI tract failure (6) explains at least in part heart failure associated with cardiac myocyte necrosis (7).

Several reports have shown the concurrence of inflammatory bowel diseases and MS [61–65]. Moreover, brain micro-inflammations were observed in patients with neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease [66, 67], epilepsy [68], and psychological disorders [69]. We therefore suggest that brain micro-inflammations could regulate the homeostasis of organ functions including the brain itself by acting as a switch to stimulate resting neural pathways, which could account for the comorbidities observed in many diseases.

2.5. Cholinergic anti-inflammatory pathway

The Tracey laboratory has demonstrated using a mouse model of sepsis that the activation of vagal nerves, which mainly consist of parasympathetic nerves, suppresses an inflammatory response [70]. They revealed that lipopolysaccharide induces norepinephrine release in the spleen via vagal and splenic nerves. The norepinephrine stimulates a novel subset of CD4+ T cells that express β1/2 adrenergic receptor and produce acetylcholine. The resulting acetylcholine then acts on activated macrophages expressing α7 nicotinic receptor to suppress the

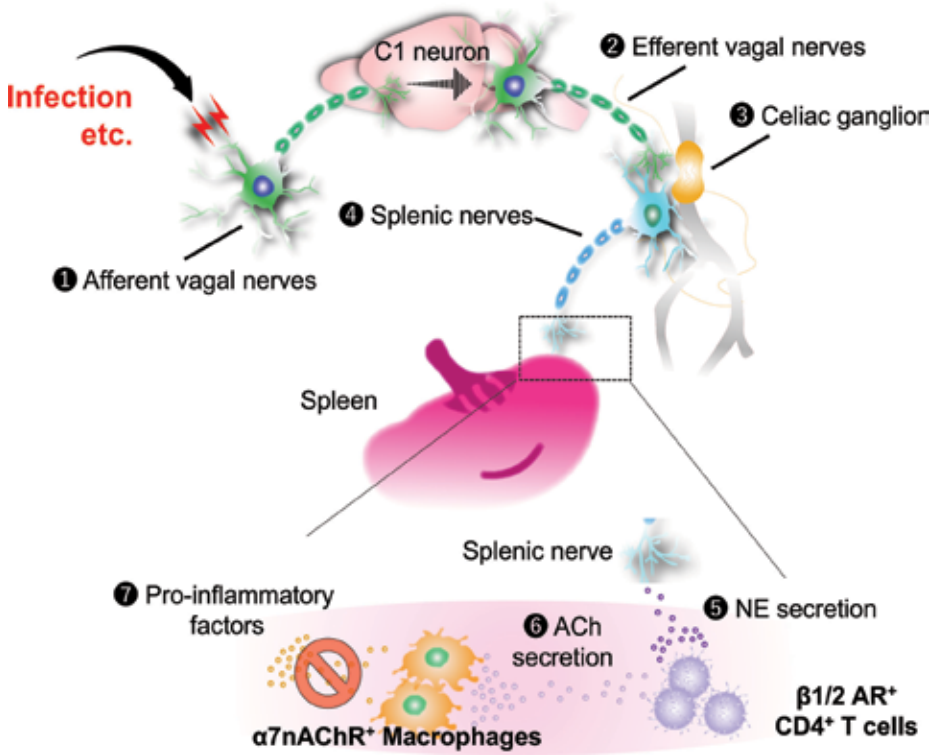

Figure 6. Inflammatory reflex. Afferent (1) and efferent vagal nerve (2) activation by infection, and so on induces neural activation in the celiac ganglion (3), followed by the production of norepinephrine (NE) by the splenic nerves (4). NE stimulates the release of acetylcholine (ACh) from a subset of CD4+ T cells expressing β1/2 adrenaline receptor (AR) (5). Then, ACh (6) acts on macrophages expressing α7 nicotinic acetylcholine receptor (α7nAChR) to suppress the expression of pro-inflammatory factors such as HMGB1 and TNFα. It is suggested that C1 neurons in the brain mediate the cholinergic anti-inflammatory effect.

expression of pro-inflammatory factors such as TNFα and HMGB1 (**Figure 6**) [71–73]. The stimulation of macrophages with nicotine inhibits NF-κB activation, but not MAP kinase activation in response to endotoxin [72]. This cholinergic anti-inflammatory pathway is called the "inflammatory reflex" [27–29, 31, 32, 34, 74–76]. In addition to infection, this pathway exerts anti-inflammatory effects in various disease models including renal ischemia–reperfusion injury, acute kidney injury, pressure overload-induced cardiac hypertrophy and neointimal hyperplasia [77–81]. The direct stimulation of C1 neurons in the medullary reticular formation induced this anti-inflammatory effect in mice [79], suggesting the involvement of C1 neurons between afferent and efferent vagal nerves. Activation of the cholinergic anti-inflammatory pathway can be induced by acupuncture and ultrasound, thus inhibiting the pathology of animal models [82, 83]. These findings establish a scientific basis for acupuncture and physical therapy. In addition, activation of the inflammatory reflex through vagal nerve stimulation by an implantable device has been tested in humans for chronic inflammatory diseases including rheumatoid arthritis with promising results [84, 85].

http://dx.doi.org/10.5772/intechopen.77112

2.6. Future directions

Accumulating evidence has demonstrated the significant effects of regional neuro-immune interactions on organ homeostasis, particularly during inflammation and diseases. The gateway reflex and inflammatory reflex can be induced by various stimulations to activate these interactions. Stimulation of the vagal nerves by an implantable device has already shown an anti-inflammatory effect in humans [84, 85]. The stimulation of the neurons responsible for the gateway reflex and inflammatory reflex at the body surface, as is the case with acupuncture, could lead to therapies that are less invasive and less costly, although elucidation of the precise neural circuits activated by the reflexes is required. Neural mapping is still challenging, but recent techniques and tools including the tissue clearing method CUBIC [86–89], optogenetics and chemogenetics [90, 91], and transgenic mice reporting neural circuitry and activations [92–94] will greatly contribute. Because neural circuits run throughout the body and because nonimmune cells as well as immune cells [18, 19, 71, 95–97] are able to secrete and respond to neurotransmitters, specific regional neuro-immune interactions such as the gateway reflex and inflammatory reflex bear potential as a significant therapeutic strategy to recover organ homeostasis.

Acknowledgements

We appreciate the outstanding experimental contributions provided by Dr. Y. Arima and the excellent technical assistances by Ms. Ezawa and Ms. Nakayama for our work described here. We also thank Ms. Fukumoto for her excellent secretarial assistance, and Dr. P. Karagiannis (CiRA, Kyoto University, Kyoto, Japan) for carefully reading the manuscript. Our work described here was supported by KAKENHI (D. K. and M. M.), Takeda Science Foundation (M. M.), Institute for Fermentation Osaka (M. M.), Mitsubishi Foundation (M. M.), Mochida Memorial Foundation for Medical and Pharmaceutical Research (D. K.), Suzuken Memorial Foundation (D. K.), and Tokyo Medical Research Foundation (M. M.).

Conflict of interest

The authors have no conflict of interest for the article.

Author details

Daisuke Kamimura, Yuki Tanaka, Takuto Ohki and Masaaki Murakami*

*Address all correspondence to: murakami@igm.hokudai.ac.jp

Molecular Psychoimmunology, Institute for Genetic Medicine, Graduate School of Medicine, Hokkaido University, Sapporo, Hokkaido, Japan

References

[1] Petersen N, Lambrecht G, Scott J, Hirsch N, Stokes M, Mester J. Postflight reconditioning for European astronauts—A case report of recovery after six months in space. Musculoskeletal Science & Practice. 2017;**27**(Suppl 1):S23-S31. DOI: 10.1016/j.msksp.2016.12.010

[2] Sibonga JD. Spaceflight-induced bone loss: Is there an osteoporosis risk? Current Osteoporosis Reports. 2013;**11**(2):92-98. DOI: 10.1007/s11914-013-0136-5

[3] De Bosscher K, Vanden Berghe W, Haegeman G. Mechanisms of anti-inflammatory action and of immunosuppression by glucocorticoids: Negative interference of activated glucocorticoid receptor with transcription factors. Journal of Neuroimmunology. 2000;**109**(1):16-22

[4] Bellavance MA, Rivest S. The HPA-immune axis and the immunomodulatory actions of glucocorticoids in the brain. Frontiers in Immunology. 2014;**5**(136). DOI: 10.3389/fimmu.2014.00136

[5] Ransohoff RM, Kivisakk P, Kidd G. Three or more routes for leukocyte migration into the central nervous system. Nature Reviews Immunology. 2003;**3**(7):569-581. DOI: 10.1038/nri1130

[6] Steed E, Balda MS, Matter K. Dynamics and functions of tight junctions. Trends in Cell Biology. 2010;**20**(3):142-149. S0962-8924(09)00297-9 [pii]. DOI: 10.1016/j.tcb.2009.12.002

[7] Zlokovic BV. The blood-brain barrier in health and chronic neurodegenerative disorders. Neuron. 2008;**57**(2):178-201. S0896-6273(08)00034-2 [pii]. DOI: 10.1016/j.neuron.2008.01.003

[8] Tsao N, Hsu HP, Wu CM, Liu CC, Lei HY. Tumour necrosis factor-alpha causes an increase in blood-brain barrier permeability during sepsis. Journal of Medical Microbiology. 2001;**50**(9):812-821

[9] Argaw AT, Zhang Y, Snyder BJ, Zhao ML, Kopp N, Lee SC, et al. IL-1beta regulates blood-brain barrier permeability via reactivation of the hypoxia-angiogenesis program. Journal of Immunology. 2006;**177**(8):5574-5584

[10] Nagarsheth N, Wicha MS, Zou W. Chemokines in the cancer microenvironment and their relevance in cancer immunotherapy. Nature Reviews Immunology. 2017;**17**(9):559-572. DOI: 10.1038/nri.2017.49

[11] Keegan BM, Noseworthy JH. Multiple sclerosis. Annual Review of Medicine. 2002;**53**:285-302. DOI: 10.1146/annurev.med.53.082901.103909

[12] McFarland HF, Martin R. Multiple sclerosis: A complicated picture of autoimmunity. Nature Immunology. 2007;**8**(9):913-919. DOI: 10.1038/ni1507

[13] International Multiple Sclerosis Genetics C, Wellcome Trust Case Control C, Sawcer S, Hellenthal G, Pirinen M, Spencer CC, et al. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. Nature. 2011;**476**(7359):214-219. DOI: 10.1038/nature10251

[14] Australia, New Zealand Multiple Sclerosis Genetics C. Genome-wide association study identifies new multiple sclerosis susceptibility loci on chromosomes 12 and 20. Nature Genetics 2009;**41**(7):824-828. 10.1038/ng.396

[15] Moutsianas L, Jostins L, Beecham AH, Dilthey AT, Xifara DK, Ban M, et al. Class II HLA interactions modulate genetic risk for multiple sclerosis. Nature Genetics. 2015;**47**(10): 1107-1113. DOI: 10.1038/ng.3395

[16] Steinman L, Zamvil SS. Virtues and pitfalls of EAE for the development of therapies for multiple sclerosis. Trends in Immunology. 2005;**26**(11):565-571. DOI: 10.1016/j.it.2005.08.014

[17] Rangachari M, Kuchroo VK. Using EAE to better understand principles of immune function and autoimmune pathology. Journal of Autoimmunity. 2013;**45**:31-39. DOI: 10.1016/j.jaut.2013.06.008

[18] Arima Y, Harada M, Kamimura D, Park JH, Kawano F, Yull FE, et al. Regional neural activation defines a gateway for autoreactive T cells to cross the blood-brain barrier. Cell. 2012;(3):**148**:447-457. S0092-8674(12)00088-8 [pii]. DOI: 10.1016/j.cell.2012.01.022

[19] Arima Y, Kamimura D, Atsumi T, Harada M, Kawamoto T, Nishikawa N, et al. A pain-mediated neural signal induces relapse in murine autoimmune encephalomyelitis, a multiple sclerosis model. eLife. 2015;**4**:e08733. DOI: 10.7554/eLife.08733

[20] Tanaka Y, Arima Y, Higuchi K, Ohki T, Elfeky M, Ota M, et al. EAE induction by passive transfer of MOG-specific CD4+ T cells. Bio-protocol. 2017;**7**(13):7/5/2017. DOI: 10.21769/BioProtoc.2370

[21] Komiyama Y, Nakae S, Matsuki T, Nambu A, Ishigame H, Kakuta S, et al. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. Journal of Immunology. 2006;**177**(1):566-573

[22] Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 cells. Annual Review of Immunology. 2009;**27**:485-517. DOI: 10.1146/annurev.immunol.021908.132710

[23] Ohira Y, Kawano F, Stevens JL, Wang XD, Ishihara A. Load-dependent regulation of neuromuscular system. Journal of Gravitational Physiology. 2004;**11**:127-128

[24] Shen J, Wang HY, Chen JY, Liang BL. Morphologic analysis of normal human lumbar dorsal root ganglion by 3D MR imaging. American Journal of Neuroradiology. 2006;**27**(10):2098-2103

[25] Morey-Holton ER, Globus RK. Hindlimb unloading rodent model: Technical aspects. Journal of Applied Physiology. 2002;**92**(4):1367-1377. DOI: 10.1152/japplphysiol.00969.2001

[26] Ohira T, Kawano F, Ohira T, Goto K, Ohira Y. Responses of skeletal muscles to gravitational unloading and/or reloading. The Journal of Physiological Sciences. 2015;**65**(4): 293-310. DOI: 10.1007/s12576-015-0375-6

[27] Tracey KJ. Immune cells exploit a neural circuit to enter the CNS. Cell. 2012;**148**:392-394

[28] Andersson U, Tracey KJ. Neural reflexes in inflammation and immunity. The Journal of Experimental Medicine. 2012;**209**(6):1057-1068. DOI: 10.1084/jem.20120571

[29] Deutschman CS, Tracey KJ. Sepsis: Current dogma and new perspectives. Immunity. 2014;**40**(4):463-475. DOI: 10.1016/j.immuni.2014.04.001

[30] Sabharwal L, Kamimura D, Meng J, Bando H, Ogura H, Nakayama C, et al. The gateway reflex, which is mediated by the inflammation amplifier, directs pathogenic immune cells into the CNS. Journal of Biochemistry. 2014;**156**:299-304

[31] Tracey KJ. Reflexes in immunity. Cell. 2016;**164**(3):343-344. DOI: 10.1016/j.cell.2016.01.018

[32] Pavlov VA, Tracey KJ. Neural regulation of immunity: Molecular mechanisms and clinical translation. Nature Neuroscience. 2017;**20**(2):156-166. DOI: 10.1038/nn.4477

[33] Ohki T, Kamimura D, Arima Y, Murakami M. Gateway reflex, a new paradigm of neuroimmune interaction. Clinical & Experimental Neuroimmunology. 2017;**8**:23-32. DOI: 10.1111/cen3.12378

[34] Chavan SS, Pavlov VA, Tracey KJ. Mechanisms and therapeutic relevance of neuroimmune communication. Immunity. 2017;**46**(6):927-942. DOI: 10.1016/j.immuni.2017.06.008

[35] Ogura H, Murakami M, Okuyama Y, Tsuruoka M, Kitabayashi C, Kanamoto M, et al. Interleukin-17 promotes autoimmunity by triggering a positive-feedback loop via interleukin-6 induction. Immunity. 2008;**29**:628-636

[36] Atsumi T, Singh R, Sabharwal L, Bando H, Jie M, Arima Y, et al. Inflammation amplifier, a new paradigm in cancer biology. Cancer Research. 2014;**74**:8-14

[37] Nakagawa I, Kamimura D, Atsumi T, Arima Y, Murakami M. Role of inflammation amplifier-induced growth factor expression in the development of inflammatory diseases. Critical Reviews in Immunology. 2015;**35**(5):365-378

[38] Arima Y, Ohki T, Nishikawa N, Higuchi K, Ota M, Tanaka Y, et al. Brain micro-inflammation at specific vessels dysregulates organ-homeostasis via the activation of a new neural circuit. eLife. 2017;**6**:e25517. DOI: 10.7554/eLife.25517

[39] Murakami M, Okuyama Y, Ogura H, Asano S, Arima Y, Tsuruoka M, et al. Local microbleeding facilitates IL-6- and IL-17-dependent arthritis in the absence of tissue antigen recognition by activated T cells. The Journal of Experimental Medicine. 2011;**208**(1): 103-114. DOI: 10.1084/jem.20100900

[40] Lee J, Nakagiri T, Oto T, Harada M, Morii E, Shintani Y, et al. IL-6 amplifier, NF-kappaB-triggered positive feedback for IL-6 signaling, in grafts is involved in allogeneic rejection responses. Journal of Immunology. 2012;**189**(4):1928-1936. DOI: 10.4049/jimmunol.1103613

[41] Murakami M, Harada M, Kamimura D, Ogura H, Okuyama Y, Kumai N, et al. Disease-association analysis of an inflammation-related feedback loop. Cell Reports. 2013;**3**(3): 946-959. DOI: 10.1016/j.celrep.2013.01.028

[42] Lee J, Nakagiri T, Kamimura D, Harada M, Oto T, Susaki Y, et al. IL-6 amplifier activation in epithelial regions of bronchi after allogeneic lung transplantation. International Immunology. 2013;**25**(5):319-332. DOI: 10.1093/intimm/dxs158

[43] Harada M, Kamimura D, Arima Y, Kohsaka H, Nakatsuji Y, Nishida M, et al. Temporal expression of growth factors triggered by epiregulin regulates inflammation development. Journal of Immunology. 2015;**194**(3):1039-1046. DOI: 10.4049/jimmunol.1400562

[44] Meng J, Jiang JJ, Atsumi T, Bando H, Okuyama Y, Sabharwal L, et al. Breakpoint cluster region-mediated inflammation is dependent on casein kinase II. Journal of Immunology. 2016;**197**(8):3111-3119. DOI: 10.4049/jimmunol.1601082

[45] Atsumi T, Suzuki H, Jiang JJ, Okuyama Y, Nakagawa I, Ota M, et al. Rbm10 regulates inflammation development via alternative splicing of Dnmt3b. International Immunology. 2017;**29**(12):581-591. DOI: 10.1093/intimm/dxx067

[46] Kipnis J, Cohen H, Cardon M, Ziv Y, Schwartz M. T cell deficiency leads to cognitive dysfunction: Implications for therapeutic vaccination for schizophrenia and other psychiatric conditions. Proceedings of the National Academy of Sciences of the United States of America. 2004;**101**(21):8180-8185. DOI: 10.1073/pnas.0402268101

[47] Wolf SA, Steiner B, Akpinarli A, Kammertoens T, Nassenstein C, Braun A, et al. CD4-positive T lymphocytes provide a neuroimmunological link in the control of adult hippocampal neurogenesis. Journal of Immunology. 2009;**182**(7):3979-3984. DOI: 10.4049/jimmunol.0801218

[48] Derecki NC, Cardani AN, Yang CH, Quinnies KM, Crihfield A, Lynch KR, et al. Regulation of learning and memory by meningeal immunity: A key role for IL-4. The Journal of Experimental Medicine. 2010;**207**(5):1067-1080. DOI: 10.1084/jem.20091419

[49] Morales-Lazaro SL, Simon SA, Rosenbaum T. The role of endogenous molecules in modulating pain through transient receptor potential vanilloid 1 (TRPV1). The Journal of Physiology. 2013;**591**(Pt 13):3109-3121. DOI: 10.1113/jphysiol.2013.251751

[50] Bennett DL, Woods CG. Painful and painless channelopathies. The Lancet Neurology. 2014;**13**(6):587-599. DOI: 10.1016/S1474-4422(14)70024-9

[51] Feinstein A, Magalhaes S, Richard JF, Audet B, Moore C. The link between multiple sclerosis and depression. Nature Reviews Neurology. 2014;**10**(9):507-517. DOI: 10.1038/nrneurol.2014.139

[52] Ehde DM, Gibbons LE, Chwastiak L, Bombardier CH, Sullivan MD, Kraft GH. Chronic pain in a large community sample of persons with multiple sclerosis. Multiple Sclerosis. 2003;**9**:605-611

[53] Ehde DM, Osborne TL, Hanley MA, Jensen MP, Kraft G H. The scope and nature of pain in persons with multiple sclerosis. Multiple Sclerosis. 2006;**12**:629-638

[54] O'Connor AB, Schwid SR, Herrmann DN, Markman JD, Dworkin RH. Pain associated with multiple sclerosis: Systematic review and proposed classification. Pain. 2008;**137**(1):96-111. DOI: 10.1016/j.pain.2007.08.024

[55] Lu J, Kurejova M, Wirotanseng LN, Linker RA, Kuner R, Tappe-Theodor A. Pain in experimental autoimmune encephalitis: A comparative study between different mouse models. Journal of Neuroinflammation. 2012;**9**:233. DOI: 10.1186/1742-2094-9-233

[56] Khan N, Smith MT. Multiple sclerosis-induced neuropathic pain: Pharmacological man agement and pathophysiological insights from rodent EAE models. Inflammopharmacology. 2014;**22**(1):1-22. DOI: 10.1007/s10787-013-0195-3

[57] Thygesen TH, Baad-Hansen L, Svensson P. Sensory action potentials of the maxillary nerve: A methodologic study with clinical implications. Journal of Oral and Maxillofacial Surgery. 2009;**67**:537-542

[58] Ulrich-Lai YM, Herman JP. Neural regulation of endocrine and autonomic stress responses. Nature Reviews Neuroscience. 2009;**10**(6):397-409. DOI: 10.1038/nrn2647

[59] Burnstock G. Historical review: ATP as a neurotransmitter. Trends in Pharmacological Sciences. 2006;**27**(3):166-176. DOI: 10.1016/j.tips.2006.01.005

[60] Fiebich BL, Akter S, Akundi RS. The two-hit hypothesis for neuroinflammation: Role of exogenous ATP in modulating inflammation in the brain. Frontiers in Cellular Neuroscience. 2014;**8**:260. DOI: 10.3389/fncel.2014.00260

[61] Rang EH, Brooke BN, Hermon-Taylor J. Association of ulcerative colitis with multiple sclerosis. Lancet. 1982;**2**(8297):555

[62] Sadovnick AD, Paty DW, Yannakoulias G. Concurrence of multiple sclerosis and inflammatory bowel disease. The New England Journal of Medicine. 1989;**321**(11):762-763

[63] Gupta G, Gelfand JM, Lewis JD. Increased risk for demyelinating diseases in patients with inflammatory bowel disease. Gastroenterology. 2005;**129**(3):819-826. DOI: 10.1053/j.gastro.2005.06.022

[64] Kimura K, Hunter SF, Thollander MS, Loftus EV Jr, Melton LJ 3rd, O'Brien PC, et al. Concurrence of inflammatory bowel disease and multiple sclerosis. Mayo Clinic Proceedings. 2000;**75**(8):802-806. DOI: 10.4065/75.8.802

[65] Pokorny CS, Beran RG, Pokorny MJ. Association between ulcerative colitis and multiple sclerosis. Internal Medicine Journal. 2007;**37**(10):721-724. DOI: 10.1111/j.1445-5994.2007.01452.x

[66] Togo T, Akiyama H, Iseki E, Kondo H, Ikeda K, Kato M, et al. Occurrence of T cells in the brain of Alzheimer's disease and other neurological diseases. Journal of Neuroimmunology. 2002;**124**(1-2):83-92

[67] Appel SH, Beers DR, Henkel JS. T cell-microglial dialogue in Parkinson's disease and amyotrophic lateral sclerosis: Are we listening? Trends in Immunology. 2010;**31**:7-17

[68] Vezzani A, French J, Bartfai T, Baram TZ. The role of inflammation in epilepsy. Nature Reviews. Neurology. 2011;**7**:31-40

[69] Najjar S, Pearlman DM, Alper K, Najjar A, Devinsky O. Neuroinflammation and psychiatric illness. Journal of Neuroinflammation. 2013;**10**:43. DOI: 10.1186/1742-2094-10-43

[70] Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR, et al. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature. 2000;**405**:458-462

[71] Wang H, Yu M, Ochani M, Amella CA, Tanovic M, Susarla S, et al. Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. Nature. 2003;**421**(6921):384-388. DOI: 10.1038/nature01339

[72] Wang H, Liao H, Ochani M, Justiniani M, Lin X, Yang L, et al. Cholinergic agonists inhibit HMGB1 release and improve survival in experimental sepsis. Nature Medicine. 2004;**10**(11):1216-1221. DOI: 10.1038/nm1124

[73] Rosas-Ballina M, Olofsson PS, Ochani M, Valdes-Ferrer SI, Levine YA, Reardon C, et al. Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit. Science. 2011;**334**(6052):98-101. DOI: 10.1126/science.1209985

[74] Andersson U, Tracey KJ. Reflex principles of immunological homeostasis. Annual Review of Immunology. 2012;**30**:313-335. DOI: 10.1146/annurev-immunol-020711-075015

[75] Olofsson PS, Tracey KJ. Bioelectronic medicine: Technology targeting molecular mechanisms for therapy. Journal of Internal Medicine. 2017;**282**(1):3-4. DOI: 10.1111/joim.12624

[76] Tanaka Y, Arima Y, Kamimura D, Murakami M. The gateway reflex, a novel neuroimmune interaction for the regulation of regional vessels. Frontiers in Immunology. 2017;**8**:1321. DOI: 10.3389/fimmu.2017.01321

[77] Mao Y, Tokudome T, Kishimoto I, Otani K, Nishimura H, Yamaguchi O, et al. Endogenous ghrelin attenuates pressure overload-induced cardiac hypertrophy via a cholinergic anti-inflammatory pathway. Hypertension. 2015;**65**(6):1238-1244. DOI: 10.1161/HYPERTENSIONAHA.114.04864

[78] Inoue T, Abe C, Sung SS, Moscalu S, Jankowski J, Huang L, et al. Vagus nerve stimulation mediates protection from kidney ischemia-reperfusion injury through alpha7nAChR+ splenocytes. The Journal of Clinical Investigation. 2016;**126**(5):1939-1952. DOI: 10.1172/JCI83658

[79] Abe C, Inoue T, Inglis MA, Viar KE, Huang LP, Ye H, et al. C1 neurons mediate a stress-induced anti-inflammatory reflex in mice. Nature Neuroscience. 2017;**20**(5):700-707. DOI: 10.1038/nn.4526

[80] Chatterjee PK, Yeboah MM, Solanki MH, Kumar G, Xue X, Pavlov VA, et al. Activation of the cholinergic anti-inflammatory pathway by GTS-21 attenuates cisplatin-induced acute kidney injury in mice. PLoS One. 2017;**12**(11):e0188797. DOI: 10.1371/journal.pone.0188797

[81] Li DJ, Fu H, Tong J, Li YH, Qu LF, Wang P, et al. Cholinergic anti-inflammatory pathway inhibits neointimal hyperplasia by suppressing inflammation and oxidative stress. Redox Biology. 2018;**15**:22-33. DOI: 10.1016/j.redox.2017.11.013

[82] Torres-Rosas R, Yehia G, Pena G, Mishra P, del Rocio Thompson-Bonilla M, Moreno-Eutimio MA, et al. Dopamine mediates vagal modulation of the immune system by electroacupuncture. Nature Medicine. 2014;**20**(3):291-295. DOI: 10.1038/nm.3479

[83] Gigliotti JC, Huang L, Bajwa A, Ye H, Mace EH, Hossack JA, et al. Ultrasound modulates the splenic neuroimmune axis in attenuating AKI. Journal of the American Society of Nephrology. 2015;**26**(10):2470-2481. DOI: 10.1681/ASN.2014080769

[84] Koopman FA, Chavan SS, Miljko S, Grazio S, Sokolovic S, Schuurman PR, et al. Vagus nerve stimulation inhibits cytokine production and attenuates disease severity in rheumatoid arthritis. Proceedings of the National Academy of Sciences of the United States of America. 2016;**113**(29):8284-8289. DOI: 10.1073/pnas.1605635113

[85] Bonaz B, Sinniger V, Pellissier S. Vagus nerve stimulation: A new promising therapeutic tool in inflammatory bowel disease. Journal of Internal Medicine. 2017;**282**(1):46-63. DOI: 10.1111/joim.12611

[86] Susaki EA, Tainaka K, Perrin D, Kishino F, Tawara T, Watanabe TM, et al. Whole-brain imaging with single-cell resolution using chemical cocktails and computational analysis. Cell. 2014;**157**(3):726-739. DOI: 10.1016/j.cell.2014.03.042

[87] Tainaka K, Kubota SI, Suyama TQ, Susaki EA, Perrin D, Ukai-Tadenuma M, et al. Whole-body imaging with single-cell resolution by tissue decolorization. Cell. 2014;**159**(4): 911-924. DOI: 10.1016/j.cell.2014.10.034

[88] Tainaka K, Kuno A, Kubota SI, Murakami T, Ueda HR. Chemical principles in tissue clearing and staining protocols for whole-body cell profiling. Annual Review of Cell and Developmental Biology. 2016;**32**:713-741. DOI: 10.1146/annurev-cellbio-111315-125001

[89] Murakami TC, Mano T, Saikawa S, Horiguchi SA, Shigeta D, Baba K, et al. A three-dimensional single-cell-resolution whole-brain atlas using CUBIC-X expansion microscopy and tissue clearing. Nature Neuroscience. 2018;**21**:625-637. DOI: 10.1038/s41593-018-0109-1

[90] Kim CK, Adhikari A, Deisseroth K. Integration of optogenetics with complementary methodologies in systems neuroscience. Nature Reviews Neuroscience. 2017;**18**(4):222-235. DOI: 10.1038/nrn.2017.15

[91] Campbell EJ, Marchant NJ. The use of chemogenetics in behavioural neuroscience: Receptor variants, targeting approaches, and caveats. British Journal of Pharmacology. 2018;**175**:994-1003. DOI: 10.1111/bph.14146

[92] Livet J, Weissman TA, Kang H, Draft RW, Lu J, Bennis RA, et al. Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. Nature. 2007;**450**(7166):56-62. DOI: 10.1038/nature06293

[93] Eguchi M, Yamaguchi S. In vivo and in vitro visualization of gene expression dynamics over extensive areas of the brain. NeuroImage. 2009;**44**(4):1274-1283. DOI: 10.1016/j.neuroimage.2008.10.046

[94] Kebschull JM, Garcia da Silva P, Reid AP, Peikon ID, Albeanu DF, Zador AM. High-throughput mapping of single-neuron projections by sequencing of barcoded RNA. Neuron. 2016;**91**(5):975-987. DOI: 10.1016/j.neuron.2016.07.036

[95] Levite M. Neurotransmitters activate T-cells and elicit crucial functions via neurotransmitter receptors. Current Opinion in Pharmacology. 2008;**8**(4):460-471. DOI: 10.1016/j.coph.2008.05.001

[96] Selmeczy Z, Vizi ES, Csoka B, Pacher P, Hasko G. Role of nonsynaptic communication in regulating the immune response. Neurochemistry International. 2008;**52**(1-2):52-59. DOI: 10.1016/j.neuint.2007.06.001

[97] Camacho-Arroyo I, Lopez-Griego L, Morales-Montor J. The role of cytokines in the regulation of neurotransmission. Neuroimmunomodulation. 2009;**16**(1):1-12. DOI: 10.1159/000179661

Chapter 5

Platelets: From Formation to Function

Laura Twomey, Robert G. Wallace,
Philip M. Cummins, Bernard Degryse,
Sinead Sheridan, Michael Harrison, Niall Moyna,
Gerardene Meade-Murphy, Nastassia Navasiolava,
Marc-Antoine Custaud and Ronan P. Murphy

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.80924

Abstract

Platelets are small, anucleate cells that travel as resting discoid fragments in the circulation. Their average circulating life span is 8–9 days, and their formation is an elegant and finely orchestrated series of cellular processes known as *megakaryocytopoiesis* and t*hrombopoiesis*. This involves the commitment of haematopoietic stem cells, proliferation, terminal differentiation of megakaryocytic progenitors and maturation of megakaryocytes to produce functional platelets. This complex process occurs in specialised endosteal and vascular niches in the bone marrow where megakaryocytes form proplatelet projections, releasing platelets into the circulation. Upon contact with an injured blood vessel, they prevent blood loss through processes of adhesion, activation and aggregation. Platelets play a central role in cardiovascular disease (CVD), both in the development of atherosclerosis and as the cellular mediator in the development of thrombosis. Platelets have diverse roles not limited to thrombosis/haemostasis, also being involved in many vascular inflammatory conditions. Depending on the physiological context, platelet functions may be protective or contribute to adverse thrombotic and inflammatory outcomes. In this chapter, we will discuss platelets in context of their formation and function. Because of their multifaceted role in maintaining physiological homeostasis, current and development of platelet function testing platforms will be discussed.

Keywords: platelets, megakaryocytes, megakaryocytopoiesis and thrombopoiesis, thrombosis and haemostasis, cardiovascular disease, platelet function testing

1. Introduction

Efforts in coping with the socio-economic and health burden of CVD require further understanding of its aetiology and the risk factors behind it in order to develop cost-effective preventive strategies (primordial, primary, secondary and tertiary) to prevent and manage it. CVD risk factors can be classed as modifiable or non-modifiable. Modifiable risk factors included smoking, diabetes, unhealthy diet, cholesterol, physical inactivity (sedentary lifestyle and low cardiorespiratory fitness), overweight and obesity. Risk factors for CVD track from childhood into adulthood and are strong predictors of subclinical disease in early adulthood [1]. Up to 80% of CVD may be prevented if modifiable risk factors are evaded [2].

The main functions of blood are to supply oxygen and nutrients to tissues and cells, removal of waste, and regulation of pH and body temperature [3]. An average adult has approximately five litres of blood, accounting for about 7% of their body weight. Blood is composed of approximately 55% plasma; a pale yellow fluid mainly consisting of water, proteins, sugars and fat particles, and 45% blood cells. Blood cells include erythrocytes, leukocytes and platelets. Each of these cells are derived from a haematopoietic stem cell (HSC), which reside in the bone marrow and sit at the peak of a developmental hierarchy, with unique ability to self-renew and give rise to cells of all of the blood lineages [4]. In adults, nearly a trillion new blood cells are produced daily to sustain steady state in circulation [5]. In the classic model of haematopoiesis (the production of blood cells), an important bifurcation occurs between the lymphoid and myeloid branches, which then further divide into a number of progenitor cells (**Figure 1**). While the main function of red blood cells (RBCs) and white blood cells (WBCs) is oxygen transport and defence, respectively, this chapter will focus on platelets, the final product of one of the myeloid

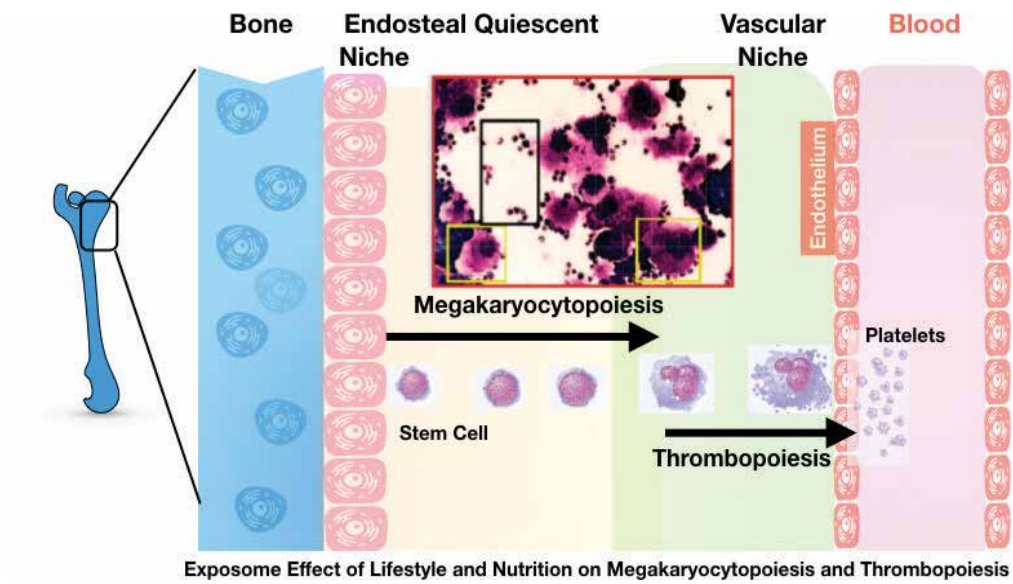

Figure 1. Pictorial representation of megakaryocytopoiesis and thrombopoiesis, and the potential for exposome and epigenome modulation of platelet phenotype and function during this dynamic cellular differentiation process.

cell lines. In addition, the principle hypothesis and paradigm discussed will be that the modifiable lifestyle factors of physical activity and inactivity can impact on the processes of both *megakaryocytopoiesis and thrombopoiesis*, *via* epigenetic mechanisms. We propose that physical activity/inactivity can modulate and program platelet phenotype and therefore function. Through recent studies, including our own (manuscripts in preparation), it is becoming increasingly evident that lifestyle factors such as physical (in)-activity and high BMI do impact on platelet function. Thus, various research studies have collectively demonstrated that platelets are indeed reflective of physiological and lifestyle changes, making them sensitive biomarkers of human health. Platelets represent a tangible link to physiological and pathological changes within the body. Future investigations will undoubtedly contribute to a greater mechanistic understanding of the relationship between cardiovascular health, lifestyle factors and platelet biology.

2. Platelets

2.1. Platelet production

Gulio Bizzozero first described platelets as 'spherules piastrine' (little plates) as small cell fragments that clumped together at an injured blood vessel site. He also showed that these blood elements did not have a nucleus [6]. Circulating anucleate platelets are now described as dynamic specialised cells, formed in an elaborate style from their precursor cell, the megakaryocyte. Normal platelet counts ranges between 150 and 450 × 10^3 per microliter of blood, constituting the second most abundant cell type in blood after red blood cells. The average adult produces 10^{11} platelets per day to preserve this count. Platelets travel as resting (quiescent) discoid fragments in the circulation, while an elaborate internal cytoskeleton allows shape changes to occur upon contact with an injured blood vessel.

The size of a mature platelet is approximately 2–4 μm, making them the smallest cells in circulation, while their average thickness is 0.5 μm [7] and their volume about 7 μm^3. Their small size facilitates in their role as 'guardians of the vasculature', as under laminar flow environments, platelets are pushed to the periphery by larger white and red blood cells. Consequently, they remain in close proximity to the blood vessel wall where they can quickly respond to any vascular damage [8]. This enables platelets to perform their main physiological function to prevent blood loss in primary haemostasis by the formation of a 'platelet plug' [9].

Platelets are formed and released into the bloodstream from megakaryocytes (MKs), which reside in the bone marrow [10, 11]. Their production is arguably the most elegant and distinct developmental process in eukaryotes [12]. While accounting for only 0.01% of nucleated bone marrow cells, MKs are also the largest cells, measuring between 50 and 100 μm [13]. Both MK and platelet production, termed megakaryocytopoiesis and thrombopoiesis, are regulated by multiple cytokines, with thrombopoietin (TPO), a hormone produced by the liver and kidneys, being the key regulator. In response to TPO, HSCs differentiate into MKs by differential expression of various transcription factors. This maturation is characterised by a growth in MK size and DNA ploidy levels (endomitosis), enabling the accumulation of RNA, protein and organelles in the MK for packaging into platelets. MKs then migrate to the sinusoidal

blood vessels in the vascular niche where numerous long processes called pro-platelets are formed. MKs can extend as many as 20 pro-platelets which branch repeatedly over time. Platelets form at the tips of pro-platelets, receiving organelles, genetic material and granule contents that are transported from the MK cell body. The final point of platelet production occurs in circulation whereby anucleate fragments of pro-platelets bud into pre-platelets [14] and barbell-shaped platelets [15] that are subsequently converted into single platelets in a microtubule-driven process [16] aided by the shear forces within the bloodstream [11]. Platelets then have an average lifespan of 8–10 days, after which they are cleared via phagocytic cells such as macrophages in the spleen. Apoptosis (programmed cell death) is also well recognised in the anucleate platelet [17]. The constant number of platelets in circulation is a consequence of a homeostatic balance between their production and destruction/clearance.

2.2. Platelet structure

2.2.1. Internal

Platelets are unique in their structural composition and, while anucleate, contain a large variety of cellular organelles, granules and mitochondria. Granules are generally secretory vesicles that release their contents either to the platelet surface or to extracellular fluid by endocytosis. Over 300 proteins from platelet granules have been identified in the platelet releasate following activation [18, 19]. Three types of platelet granules have been identified: α-granules, dense granules, and lysosomal granules all of which derive their cargo from MKs. α-granules are the largest and most numerous (50–80 per platelet) encompassing roughly 10% of the platelet volume [20]. They harbour a vast assortment of proteins important for primary haemostasis including integrins (αIIbβ3) immunoglobulin family receptors (e.g. GPVI, PECAM), leucine-rich repeat family receptors (e.g., GPIb-IX-V complex), tetraspanins (e.g., CD9) and other adhesive proteins such as von Willebrand Factor (vWF), fibrinogen, and coagulation factors (Factors V, XI) that participate in secondary haemostasis. While it was previously assumed that platelet α-granules were homogenous populations, [21] suggested that platelets have distinct subpopulations of alpha granules which differentially release their cargo in a context-dependent manner. Dense granules are smaller in size and number (3–8 per platelet) storing high concentrations of non-protein molecules that potentiate platelet activation such as adenosine diphosphate (ADP), adenosine triphosphate (ATP), calcium, histamine, polyphosphate and serotonin [22, 23]. Lysosomal granules are sparse and harbour enzymes such as acid hydrolases and proteases). They function in the digestion of cytosolic components. Secretion of the lysosomal content has key extracellular functions including receptor cleavage, fibrinolysis and degradation of extracellular matrix (ECM) [24]. A recent report has described a possible new type of secretory granule termed a T-granule, after their tubular morphology [11]. These novel electron-dense granules have been proposed to function in toll-like receptor (TLR) organisation and signalling. Platelet granule deficiencies or defects such as the Grey Platelet Syndrome (α-granule deficiency) or Hermansky-Pudlak Syndrome (dense granule deficiency) can cause mild to severe bleeding disorders [25].

Platelets contain functional mitochondria, which despite being few in number, have higher rates of ATP turnover than resting mammalian muscle, suggesting they are very metabolically

active [26]. The traditional role of mitochondria in the platelet is to the supply of energy in the form of ATP for primary platelet functions. However, novel functions for mitochondria continue to emerge. Dual activation of platelets with collagen and thrombin results in a subtype of platelets known as collagen and thrombin activated (COAT) platelets. COAT platelets display striking alterations in function and structure to typical "activated platelets" by exhibiting a myriad of features such as phosphatidylserine exposure due to cytoskeletal reorganisation, high microparticle release, and increased levels of fibrinogen on the platelet surface [27]. Mitochondrial membrane potential ($\Delta\Psi_m$) is reduced in (COAT) platelets and decreases in parallel with elevated mitochondrial ROS levels that are necessary for facilitating platelet PS exposure upon activation [28]. Mitochondria are involved in the process of platelet apoptosis [29] and can be released from platelets as potential inflammatory mediators [30].

2.2.2. Surface receptors

Platelets express a wide variety of receptors on their membrane, which are fundamental to platelet function and downstream signalling [9]. Major receptors include integrins, leucine-rich repeat receptors (Glycoprotein GPIb/IX/V, Toll-like receptors), C-type lectin receptors (P-Selectin, CLEC-2), tyrosine kinase receptors (Ephrins and Eph kinases), proteins belonging to the immunoglobulin superfamily (GPVI, FcγRIIA) and other receptors shared with vascular cells (TNF receptor type, CD63, CD36, PSGL-1).

Integrins are type I transmembrane cell adhesion receptors [31] consisting of a short intracellular and larger extracellular domain. All integrins contain an α subunit and a β subunit, capable of bi-directional signalling. During signal transduction, they transmit information concerning the chemical and mechanical status of the ECM to the cell [32, 33]. Platelets express five integrin receptors—each with affinity for specific ligands; αIIbβ3 (fibrinogen), α2β1(collagen), α5β1(fibronectin), αVβ3 (vitronectin) and α6β1 (laminin), all of which share related signal transduction processes [34]. On the cytoplasmic face of the plasma membrane, integrins regulate cytoskeletal dynamics and signalling complexes. On the extracellular side, integrins bind with high affinity to either ECM ligands or counter receptors on adjacent cell surfaces [35]. αIIbβ3 is the most abundant platelet integrin, with copy numbers of ~50,000 per platelet, and is present in both alpha granules and the platelet surface. Platelets express a number of G-protein coupled receptors (GPCRs) which constitute a large family of receptors that can identify molecules outside the cell and initiate signal transduction pathways and ultimately cell function. The main GPCRs present on platelets include thrombin receptors called protease-activated receptors (PARs) (PAR1 and PAR4), ADP receptors ($P2Y_1$, $P2Y_{12}$), of which approximately 150 $P2Y_1$ receptors are present on the platelet [36], thromboxane receptors (TPα and TPβ) and glycoprotein receptors [37].

2.3. Platelet function

2.3.1. Platelet function in primary haemostasis

Haemostasis can be subdivided into primary haemostasis, secondary haemostasis and fibrinolysis (**Figure 2**) [38]. Platelets prevent blood loss in primary haemostasis, the physiological

process which halts bleeding at an injured blood vessel, while maintaining normal blood flow elsewhere in circulation, by the formation of a 'platelet plug' [11]. Secondary haemostasis refers to the deposition of insoluble fibrin that is generated by the coagulation cascade.

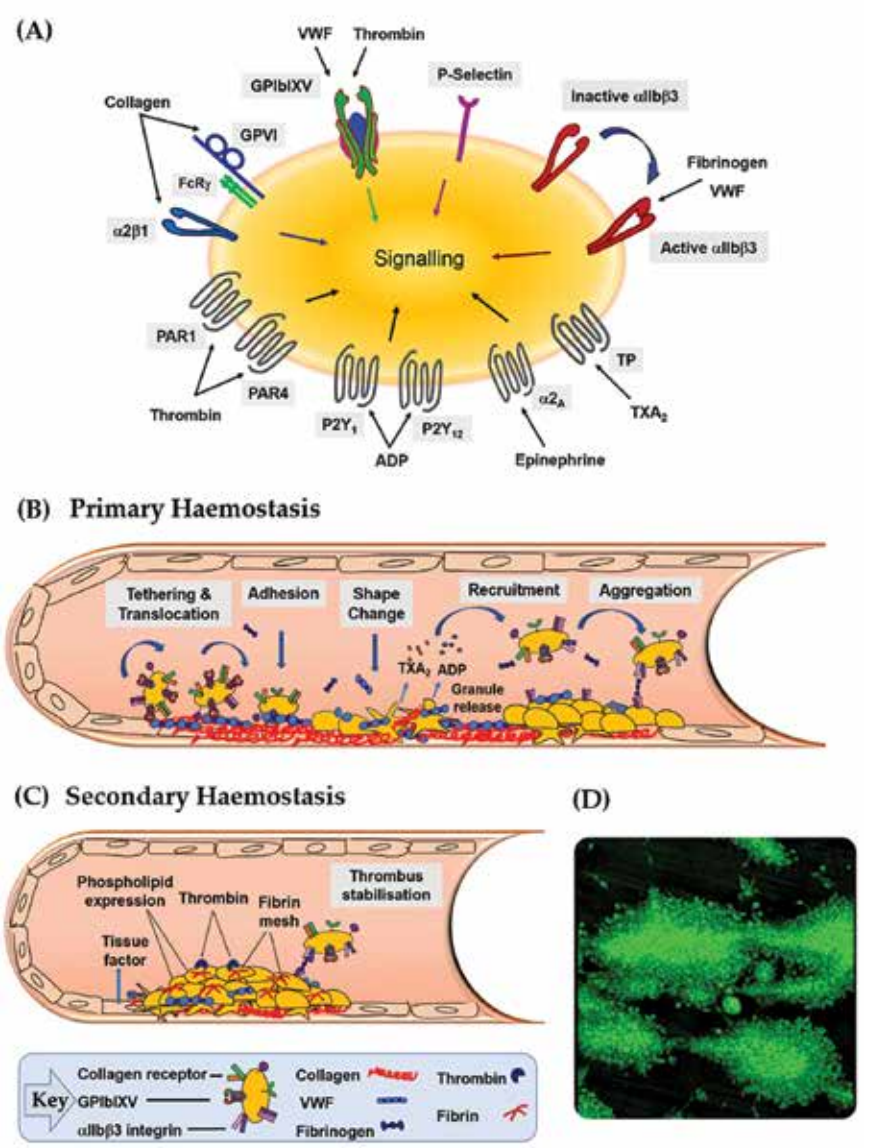

Figure 2. (A) Diagram showing key platelet membrane receptors involved in platelet function in haemostasis and thrombosis. (B) Distinct phases of platelet function in primary haemostasis: Initial platelet tethering and adhesion at a site of vascular damage, firm adhesion and shape change, activation, granule secretion and further recruitment of platelets leading to platelet aggregation. (C) Secondary haemostasis: Formation, deposition and cross-linking of insoluble fibrin, generated by the coagulation cascade, to stabilise the primary platelet plug. (D) Image of platelet aggregates (fluorescently-labelled, green) on collagen fibrils following *in vitro* blood flow at arterial shear (1800 s^{-1}) using a parallel-plate blood perfusion chamber (*unpublished data—Gerardene Meade-Murphy*).

Finally, fibrinolysis results in the breakdown of blood clots during wound healing involving the interplay of a number of enzymes [38]. A healthy endothelium provides a non-adhesive surface for platelets. However, in areas of vascular injury, the sub-endothelium is exposed and platelets may adhere quickly to different extracellular matrix components, and then form a platelet plug. This process is achieved through three distinct processes—platelet adhesion, platelet activation and secretion, and platelet aggregation [39].

2.3.2. Platelet adhesion

Platelet adhesion entails a collaborative effort of various platelet receptors, fundamentally leading to platelet activation and aggregation. ECM elements that platelets adhere to include proteins such as collagen, vWF, fibronectin, laminin and fibrinogen among others [40, 41]. Among these subendothelial substrates, the thrombogenic fibrillar collagens type I and III are the most powerful intermediaries of platelet adhesion due to their robust activating potential and affinity for vWF [42]. Following vascular damage, initial platelet 'tethering' is mediated by the interaction between the A1 domain of vWF deposited in the subendothelial matrix of the damaged vessel wall, and the GPIbα in the platelet receptor GPIb-IX-V. This interaction is particularly important at high shear rates supporting platelet translocation (i.e. decelerating platelets and keeping them in close contact with the endothelium) over the subendothelium, but not stable adhesion [43]. This interaction allows engagement of other platelet receptors. vWF/GPIb-IX interface also induces platelet activation signalling events, resulting in integrin activation [44].

Following platelet tethering, platelet collagen receptors, GPVI and α2β1 interact with exposed collagen and promote platelet adhesion and activation. GPVI is non-covalently coupled to the Fc Receptor chain (FcRγ) [45, 46] and has been acknowledged as the major signalling receptor for collagen. FcRγ has an immunoreceptor tyrosine-based activation motif (ITAM) on its cytoplasmic sphere. After collagen binding to GPVI, the ITAM motif on the GPVI/FcRγ complex is phosphorylated, resulting in activation of the Syk kinase pathways that phosphorylate downstream targets, ultimately resulting in increased cytosolic Ca^{2+} and subsequent platelet shape change, granule secretion and integrin activation. GPVI has a low affinity for collagen, rendering it unable to mediate stable adhesion alone. The α2β1 integrin then maintains stable adhesion to collagen. α2β1 stimulates downstream steps indirectly by reinforcing GPVI-collagen interactions [41, 47] and by direct signalling leading to activation of αIIbβ3. Platelet Ca^{2+} signalling is markedly dissimilar between GPVI and α2β1 suggesting that the alliance regarding GPVI and α2β1 supports optimal platelet adhesion.

The final step of platelet adhesion occurs via binding of platelets to other ECM components such as fibronectin, laminin and immobilised vWF. Platelets bind to fibronectin via the α5β1 receptor and αIIbβ3, whilst adhesion to laminin is mediated by their α6β1 receptor. Stable binding of platelets elicits activation pathways involving tyrosine kinases and signal transduction GPCR receptor signalling, cumulatively resulting in elevated cytosolic Ca^{2+} levels, cytoskeletal reorganisation and integrin activation.

2.3.3. Platelet activation and secretion

Once platelet adhesion has occurred at the site of vessel wall damage, platelet activation needs to be maintained for haemostasis to continue. Essential for the amplification of platelet

activation is the production and release of soluble agonists at the site of damage [26], which act in an autocrine and paracrine manner to amplify platelet activation and recruit further circulating platelets. These agonists consist of TxA_2, ADP, epinephrine and thrombin. ADP is secreted from platelet dense granules and binds to its relevant receptors, $P2Y_{12}$ and $P2Y_1$ on the platelet surface [48]. ADP is also released from red blood cells at the site of vascular damage [37]. Binding of ADP initiates a full complement of activation events such as elevation of intracellular platelet Ca^{2+}, TxA_2 synthesis, protein phosphorylation, shape change, granule release, and most importantly, activation of αIIbβ3 [49]. $P2Y_{12}$ is also the target of a class of antiplatelet drugs called thienopyridines (ticlopidine, clopidogrel, prasugrel), widely used in the prevention of vascular events in patients with CVD.

TxA_2 is a potent platelet agonist synthesised from arachidonic acid through the COX pathway and TxA_2 synthase enzymes. It subsequently binds to TPα and TPβ receptors that differ in their cytoplasmic tails, causing vasoconstriction, shape change, protein phosphorylation, secretion and platelet aggregation [50, 51]. Indeed, high levels of TxA_2 have been implicated in CVD, whilst inhibition of TxA_2 synthesis through aspirin-mediated COX inhibition is a major anti-platelet target.

The agonist thrombin rapidly accumulates at sites of vascular damage and has major functions in promoting and stabilising thrombus formation. Platelets release factors that support the activation of prothrombin, which after a complex series of sequential events in the coagulation cascade, results in the generation of thrombin [52]. The increase in cytosolic Ca^{2+} after platelet activation results in platelet phosphatidylserine (PS) exposure on the activated platelet membrane providing a procoagulant surface for thrombin to interact with its PAR1 and PAR4 (G protein-coupled) receptors.

Uniquely, thrombin activates its PAR-receptors by cleaving an N-terminal part at a consensus site. Cleavage exposes a new binding site that acts as a ligand to activate the receptor. Thrombin is the most powerful platelet activator, initiating an entire complement of platelet responses (shape change, granule secretion, TxA_2 synthesis, aggregation etc.) [53]. Thrombin can activate platelets at extremely low concentrations and within seconds, increases cytosolic level of Ca^{2+}, eliciting downstream signalling events. Unlike ADP, TxA_2 or thrombin, the catecholamine epinephrine is a weak agonist unable to cause shape change alone. However, it works collectively with the other agonists increasing their potential to activate. Epinephrine's mode of action is to inhibit cAMP formation by the platelet α_2A-adrenergic receptor [54].

2.3.4. Platelet aggregation

The ultimate step in primary haemostasis is platelet aggregation, caused by crosslinking of αIIbβ3 on adjacent platelets by fibrinogen (**Figure 2**). While platelet aggregation is a complex process involving different receptors (αIIbβ3 and GPIbα) and ligands (fibrinogen, fibronectin, and vWF), the main process involves the integrin αIIbβ3. On resting platelets, integrin αIIbβ3 has a low affinity for its ligands fibrinogen and vWF, which dramatically increases upon platelet activation. Binding of the main agonists to their respective receptors induce intracellular signals that disrupt the complex between the cytoplasmic tails of αIIbβ3. This ultimately leads to a conformational change in its extracellular globular head domains from a

low affinity resting state to a high affinity activated state in order to bind extracellular ligands such as fibrinogen and vWF. Irreversible activation of αIIbβ3 is a prerequisite for the development of irreversible platelet aggregates. Due to the symmetrical nature of fibrinogen, platelets can be 'bridged' and platelet aggregates are formed [40]. While αIIbβ3 is the major player in platelet aggregation, couplings between other platelet receptors and their ligands could be incorporated in aggregation. Some of these comprise CD40 ligand [55] interaction with αIIbβ3, the vWF-GPIb complex [56], and an involvement of fibronectin in stabilising platelet aggregation [57, 58]. Cadherin-6 was recently acknowledged as a new counter-receptor for αIIbβ3, involved in platelet aggregation [59].

Bi-directional 'inside out' and 'outside in' signals are transferred by both integrin subunits, mediating receptor conformation and platelet function [44]. After platelet stimulation with ADP, the signal from the ADP receptors is conducted to the intracellular domain of the cytoplasmic tail of αIIbβ3 and subsequently transmitted through a series of events to the extracellular domain (inside-out) causing a conformational change in the extracellular domain that binds to its ligand. Inside-out signalling necessitates binding of talin and kindlins to the cytoplasmic domain of β3. After ligand binding, a signal is sent to the cell (outside-in) to control platelet function such as filopodia and lamellipodia extension to dull platelet spreading [34, 44]. Furthermore, αIIbβ3 outside-in signals can also act as a break to curb excessive platelet activation by activated SHIP-1 [60].

2.3.5. Bio rheological factors in platelet aggregation

A significant factor influencing platelet aggregation is the distinct shear environment experienced within the vascular system. Platelets are subjected to fluctuating haemodynamic conditions *in vivo* such as shear stress and shear rate. The latter refers to the rate of increase of blood flow velocity, whereas the former denotes the force per unit area on the vessel wall [61]. Shear rates experienced by platelets range from slow flow in veins (shear rate 10 s^{-1} to 500 s^{-1}) to small arteries (approx. 2000 s^{-1}) to diseased or pathological arteries, where extremely high shear rates (up to 40,000) have been described [61, 62]. Increasing shear rate activates platelets itself. At low shear rates (<1000 s^{-1}), platelet aggregation is primarily facilitated by αIIbβ3-fibrinogen interactions. At shear rates typically over 5000 s^{-1} (but between 1000 and 10,000), a two-step sequential process occurs. The first depends on the adhesive properties of GP1bα and αIIbβ3 and is facilitated by the formation of reversible platelet aggregates. The second relies on the generation of platelet agonists and involves the irreversible activation of αIIbβ3 to form stable aggregates [63]. Accordingly, both fibrinogen and vWF, and receptors GPIbα and αIIbβ3, have distinctive but complementary roles in platelet aggregation subject to the haemodynamic environment.

2.3.6. Signal transduction during platelet function

The role of platelets in haemostasis is reliant on the equilibrium between activatory and inhibitory signals [64]. Inhibitory signals from the vasculature prevent platelet activation in healthy vessels. Activatory signals present at an injured blood vessel initiate platelet activation, and are managed by endogenous negative signalling regulators. Negative regulators

and pathways include the ITIM containing receptors that are postulated to reduce activation of PLC, PI3K and integrin αIIbβ3 [64]. The Wnt-β-catenin pathway has recently gained attention as negative regulator of platelet function [65, 66]. Wnt3a is one of these glycoproteins, which is secreted from activated platelets. It has been suggested to activate the canonical Wnt-β-catenin pathway as constituents of this pathway have been identified in platelets. The regulation of small GTPases such as Rap1, Rac1, RhoA and Cdc42 has been suggested as players in this inhibition of platelet function [65]. These pathways are less well characterised than the classical activation pathways [67]. The primary platelet inhibiting signals produced by healthy ECs are nitric oxide and prostacyclin. Both NO and prostacyclin relax blood vessels and prevent platelet activation [68]. NO is synthesised from several cells including platelets, ECs and RBCs and plays vital roles in maintaining platelets in a resting state [69–71]. Prostacyclin (PGI_2) is a physiological anti-aggregating agent produced constitutively by ECs as a result of arachidonic acid metabolism by cyclooxygenase (COX) enzymes.

Following endothelial damage, endogenous inhibitory signals are overcome and platelets react rapidly to limit blood loss. The activating stimuli, such as collagen, vWF, ADP, TxA_2 and thrombin, which induce platelet adhesion, activation and aggregation ultimately regulate a central set of signalling mediators that support activation. Three principle mediator families of platelet activation are phospholipase C (PLC), protein kinase C (PKC) and phosphatidylinositide-3-kinase (PI3K), and underlie two crucial events in platelet activation—secretion of amplifying mediators and activation of integrin αIIbβ3.

2.4. Platelet function beyond haemostasis

Platelets are recognised as pivotal players in numerous other processes ranging from inflammation and atherosclerosis, fighting microbial infection and tumour growth and metastasis. Platelets are equipped to influence inflammation, the innate immune response and infection, by acting as sentinels in pathogen detection. They express a collection of pattern recognition receptors called toll-like receptors (TLRs) that identify molecular motifs called pathogen associated molecular patterns (PAMPs) and initiate immune responses [72]. Platelets express functional TLR 1–9, whilst TLR2 stimulation in platelets by bacteria through the activation of the PI3K signalling pathway induces a pro-inflammatory response [20]. Platelet TLR9 activation has been associated with thrombosis and oxidative stress, and is found within the T-granules in platelets [11].

Platelets interact with leukocytes, monocytes and granulocytes through different receptor-ligand interactions (P-Selectin and PSGL-1 interaction) enabling intercellular communication [73]. The capacity of platelets to store and release copious inflammatory cytokines and chemokines is intimately associated to their role in inflammation. Platelets release microparticles linked to inflammatory pathways and which are associated with inflammatory diseases such as rheumatoid arthritis [74]. Platelets play key roles in infection and immune response to bacterial and viral infections. They are the first cell type to arrive at areas of vascular infection [75]. Thrombocytopenia (low platelet count) is a well-established manifestation of sepsis and studies suggests platelets play a functional role in the pathogenesis of sepsis and multi-organ failure [76].

Platelets have significant roles in the pathogenesis of metastasis [77]. Tumour cells can aggregate platelets *in vitro* and it has been proposed that platelet adhesion to metastatic cells can act as a 'cloak' around circulating tumour cells therefore acting as a shield for immune clearance. This phenomenon of platelet cloaking has resulted in pro-survival, pro-angiogenic and epithelial mesenchymal transition (EMT) in cancer cells [78]. Platelets also release growth factors such as VEGF and PDGF that can expedite tumour growth [79]. Platelets are deeply implicated in wound healing and bone health, and indeed the use of platelet rich plasma (PRP) therapy (i.e. rich in growth factors and bioactive substances) is effective in osteoarthritis [10] and in muscle damage such as rotator cuff tendinopathy [80].

2.5. Assessing function: platelet function tests (PFT)

The different functions of platelets may be reliably detected with a wide spectrum of tests (**Table 1**). These can be utilised to identify inherited or acquired platelet dysfunction, monitor antiplatelet therapy, manage various aspects of platelet banking and transfusion, and to aid in the understanding of platelet physiology in basic research. PFTs are centred around principles of platelet function such as platelet adhesion and aggregation, platelet function under shear conditions, and measurement of the platelet releasate [81]. Platelet function testing began with the evaluation of the bleeding time (the time taken for platelets to occlude an *in vivo* wound), using the Duke procedure [82], before the development of light transmission aggregometry (LTA) revolutionised the study of platelet function. Considered the historical gold standard, LTA is a relatively easy technique that involves stirring a suspension of platelet rich plasma in a cuvette in the presence of a platelet agonist (such as ADP or collagen). The cuvette is placed between a light source and photocell. Agonist addition causes *in vitro* platelet aggregation and changes in light absorbance, which is detected by the photocell [83].

Investigation of platelet function in the environmental *milieu* of whole blood under conditions that take into account most of the physiological parameters that influence platelet adhesion and aggregation (red blood cells, white blood cells, plasma) is important. The PFA-100, The Impact-R Cone and Plate analyser and the global thrombosis test are examples of such assays. The PFA-100 assesses platelet aggregation under high shear where platelets are activated in whole blood by an amalgamation of high shear stress (5000–6000 s^{-1}) and agonists (e.g. collagen and ADP), resulting in closure of an aperture [84]. The Impact-R Cone and Plate analyser is a point-of-care (POC) device which measures global platelet function by testing platelet adhesion and aggregation in whole blood, under arterial shear conditions [85, 86]. In this assay, platelet adhesion is dependent on plasma proteins vWF, fibrinogen and RBCs and WBCs. The addition of platelet agonists such as arachidonic acid (AA) and ADP in the system has enabled the evaluation of dual anti-platelet therapy [87–89]. The system is effective in the assessment of platelet function disorders in adults, [90] children [91] and new-borns [92].

2.6. Platelet indices

Platelet indices are useful as inexpensive non-invasive biomarkers for assessing platelet activation [93]. Platelet indices are straightforwardly measured by semi-automated counters in complete blood counts (CBC) and usually include four factors; platelet count (PLT), mean

Method	Sample	Principle
Platelet aggregation assays		
Light transmission aggregometry (LTA)	Citrated PRP	Measurement of light transmission in response to agonist-induced platelet activation
Impedance Aggregation	Citrated WB	Monitors changes in electrical impedance in relation to agonist-induced platelet aggregation. Whole blood specimen diluted 1:1 with physiologic saline, prior to testing
VerifyNow	Citrated WB	Turbidimetric optical detection of platelet aggregation in whole blood in response to agonists/inhibitors
Plateletworks	Citrated WB	Platelet counting pre- and post-activation
Assays measuring release reactions		
Lumi-aggregometry	Citrated WB	Combination of LTA/WBA with nucleotide release
Soluble platelet release markers (Txb2, PF4, BTF, scD40L)	Urine, serum, citrated plasma	Typically measured by ligand binding ELISA immunoassays
Shear-based assays		
PFA-100/200	Citrated WB	High-shear platelet adhesion and aggregation during formation of a platelet plug
Impact R cone and plate analyser	Citrated WB	Shear-induced platelet adhesion-aggregation upon specific surface
Global thrombosis test	Native WB	High-shear platelet plug formation—measurement of time cessation of WB flow
Platelet activation based assays		
VASP phosphorylation	Citrated WB	Flow cytometry or ELISA measurement of VASP phosphorylation
Flow cytometry	Citrated WB, PRP, washed platelets	Measurement of platelet glycoproteins, and activation markers; platelet leukocyte aggregates, platelet microvesicles

Typical platelet function tests range from assessment of their primary haemostatic function including measurement of granule secretion using lumi-aggregometry to whole blood shear based assays, which measure platelet function under flow conditions. Platelet function can be tested in washed platelets, whole blood or platelet rich plasma.

Table 1. Platelet function tests.

platelet volume (MPV), platelet distribution width (PDW), plateletcrit (PCT), and depending on the analyser, platelet large cell ratio (PLCR). PLT is a universal indicator of haemostasis in a clinical setting and is utilised as a sensitive biomarker for a range of diseases. High PLT, even within the physiological range of 150–450 μl, is associated with a greater risk of thrombosis and CVD suggesting that enhanced PLT encourage platelet hyperactivity and a pro-inflammatory state [94]. However, the consequence of high platelet numbers that are still within physiologic ranges remains unclear [95].

The indices MPV, PDW and PLCR are quantitative measures of the variability in platelet size. MPV reflects the average platelet size while PDW reflects the volume variability in platelet size

[94, 96]. The volume of circulating platelets is heterogeneous with subsequent functional differences. Some authors suggest that larger platelets are metabolically more active than smaller platelets, that they have faster rates of aggregation and release higher quantities of pro-thrombotic elements such as TxA_2 and ADP [97]. MPV and PDW levels can be altered in several diseases including T2DM [98], CVD and atherosclerosis [93] (Berger et al., 2010), and in this regard they have been suggested as markers of subclinical platelet activation. PLCR and PCT may serve as sensitive biomarkers of platelet health [99]. PLCR indicates the percentage of large platelets present in blood [93]. PLCR is significantly higher in subjects with dyslipidaemia compared to healthy subjects [100] and higher in children with T2DM compared to healthy children. Moreover, Rechcinski et al., have hypothesised that PLCR has the potential to be a prognostic biomarker [101]. Importantly, thrombogenicity of large platelets may put individuals at higher risk of acute cardiovascular events. PCT is the volume of blood occupied by platelets as a percentage, similar to the erythrocyte measurement of haematocrit (HCT). PCT reflects total platelet mass and is calculated as $PLT \times MPV/10^7$, providing comprehensive information about platelet activity. PCT has been proposed as a novel predictor of cardiovascular risk and higher PCT is associated with the risk of re-infarction and long-term mortality in CVD patients [102]. However, the clinical significance, reference values and efficacy of some of these parameters are still under exploration.

In peripheral blood, there is ample interplay between RBCs, WBCs and platelets [103] and altered levels of blood cells and their morphology have been associated with CVD [104]. Platelet adhesion and aggregate size, is influenced by platelet indices, RBC and WBC [86]. RBCs encourage platelets towards the vessel wall [105], which can affect platelet adhesion and aggregation. In this context, it is important to investigate the associations between the various indices of each blood cell to interpret the multicellular contribution to both thrombogenesis and CVD risk.

3. Conclusion

Novel techniques continue to emerge and develop the knowledge surrounding the platelet function regulation. The modern "omics" revolution enables simultaneous quantification of hundreds of molecules (e.g. protein or mRNA) from a single sample and their signatures may be reflective of platelet function changes. The amalgamation of transcriptomic and proteomic data and subsequent bioinformatic analysis will lead to a more complete characterisation of platelet function in response to environmental stimuli [106, 107]. Epigenetics and its ancillary elements, including platelet secreted microvesicles (MVs), and microRNA (miRNA), and regulation of the platelet mitochondrial genome are new avenues of investigation and testing in platelet research [108–110].

Acknowledgements

This work was supported through the Irish Higher Education Authority Programme for Research in Third Level Institutes (HEA-PRTLI Cycle 4: T3 Targeted Therapeutics & Theranostics), the Health Research Board of Ireland (HRB-RP/2005/184, HRA/2009/122, HRA/2009/122/R),

Science Foundation Ireland (SFI-04/BR/B0577, 11/TIDA/B1927) to Dr. R.P. Murphy, and the International Visitor Award, Dublin City University, Dublin, Ireland to Drs. B. Degryse and R.P. Murphy. R.P. Murphy is a management committee member of the EU H2020 COST action-CM1406, EpiChemBio. The ground based protocol and studies of microgravity (3-Day Dry Immersion) were undertaken in MEDES, The Institute for Space Medicine and Physiology, *Toulouse* University Hospital, France and was supported and funded by the Centre National d'Etudes Spatiales (*CNES*—National Centre for Space Studies) to M-A.C., R.P.M., N.N., L.T.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Author details

Laura Twomey[1], Robert G. Wallace[1], Philip M. Cummins[2], Bernard Degryse[1], Sinead Sheridan[3], Michael Harrison[4], Niall Moyna[1], Gerardene Meade-Murphy[5], Nastassia Navasiolava[6], Marc-Antoine Custaud[6] and Ronan P. Murphy[1]*

*Address all correspondence to: ronan.murphy@dcu.ie

1 School of Health and Human Performance, Dublin City University, Dublin, Ireland

2 School of Biotechnology, Dublin City University, Dublin, Ireland

3 School of Public Health, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong

4 School of Health Sciences—Health Sport and Exercise Science, Waterford Institute of Technology, Ireland

5 Department of Pharmacology and Therapeutics, University College Cork, Cork, Ireland

6 Centre de Recherche Clinique du CHU d'Angers, Angers, France

References

[1] Juhola J, Magnussen CG, Viikari JS, Kahonen M, Hutri-Kahonen N, Jula A, et al. Tracking of serum lipid levels, blood pressure, and body mass index from childhood to adulthood: The cardiovascular risk in young Finns study. The Journal of Pediatrics. 2011;**159**(4):584-590

[2] McNeal CJ, Dajani T, Wilson D, Cassidy-Bushrow AE, Dickerson JB, Ory M. Hypercholesterolemia in youth: Opportunities and obstacles to prevent premature atherosclerotic cardiovascular disease. Current Atherosclerosis Reports. 2010;**12**(1):20-28

[3] Ruggeri ZM. Platelet adhesion under flow. Microcirculation (New York, NY: 1994). 2009;**16**(1):58-83

[4] Woolthuis CM, Park CY. Hematopoietic stem/progenitor cell commitment to the megakaryocyte lineage. Blood. 2016;**127**(10):1242-1248

[5] Huang H, Cantor AB. Common features of megakaryocytes and hematopoietic stem cells: What's the connection? Journal of Cellular Biochemistry. 2009;**107**(5):857-864

[6] Roy GC. Micro-organisms or granules of Bizzozero in the blood. The Indian Medical Gazette. 1882;**17**(4):110

[7] Xu XR, Zhang D, Oswald BE, Carrim N, Wang X, Hou Y, et al. Platelets are versatile cells: New discoveries in hemostasis, thrombosis, immune responses, tumor metastasis and beyond. Critical Reviews in Clinical Laboratory Sciences. 2016;**53**(6):409-430

[8] McFadyen JD, Kaplan ZS. Platelets are not just for clots. Transfusion Medicine Reviews. 2015;**29**(2):110-119

[9] Cimmino G, Golino P. Platelet biology and receptor pathways. Journal of Cardiovascular Translational Research. 2013;**6**(3):299-309

[10] Patel S, Dhillon MS, Aggarwal S, Marwaha N, Jain A. Treatment with platelet-rich plasma is more effective than placebo for knee osteoarthritis: A prospective, double-blind, randomized trial. The American Journal of Sports Medicine. 2013;**41**(2):356-364

[11] Thon JN, Peters CG, Machlus KR, Aslam R, Rowley J, Macleod H, et al. T granules in human platelets function in TLR9 organization and signaling. The Journal of Cell Biology. 2012;**198**(4):561-574

[12] Simon LM, Edelstein LC, Nagalla S, Woodley AB, Chen ES, Kong X, et al. Human platelet microRNA-mRNA networks associated with age and gender revealed by integrated plateletomics. Blood. 2014;**123**(16):e37-e45

[13] Machlus KR, Italiano JEJ. The incredible journey: From megakaryocyte development to platelet formation. The Journal of Cell Biology. 2013;**201**(6):785-796

[14] Thon JN, Italiano JE. Platelet formation. Seminars in Hematology. 2010;**47**(3):220-226

[15] Schwertz H, Weyrich AS. Platelet precursors display bipolar behavior. The Journal of Cell Biology. 2010;**191**(4):699-700

[16] Poulter NS, Thomas SG. Cytoskeletal regulation of platelet formation: Coordination of F-actin and microtubules. The International Journal of Biochemistry & Cell Biology. 2015;**66**:69-74

[17] Gyulkhandanyan AV, Mutlu A, Freedman J, Leytin V. Selective triggering of platelet apoptosis, platelet activation or both. British Journal of Haematology. 2013;**161**(2):245-254

[18] Coppinger JA, Cagney G, Toomey S, Kislinger T, Belton O, McRedmond JP, et al. Characterization of the proteins released from activated platelets leads to localization of novel platelet proteins in human atherosclerotic lesions. Blood. 2004;**103**(6):2096-2104

[19] Wijten P, van Holten T, Woo LL, Bleijerveld OB, Roest M, Heck AJ, et al. High precision platelet releasate definition by quantitative reversed protein profiling—Brief report. Arteriosclerosis, Thrombosis, and Vascular Biology. 2013;**33**(7):1635-1638

[20] Blair P, Flaumenhaft R. Platelet alpha-granules: Basic biology and clinical correlates. Blood Reviews. 2009;**23**(4):177-189

[21] Italiano JE Jr, Battinelli EM. Selective sorting of alpha-granule proteins. Journal of Thrombosis and Haemostasis. 2009;**7**(Suppl 1):173-176

[22] Morrell CN, Aggrey AA, Chapman LM, Modjeski KL. Emerging roles for platelets as immune and inflammatory cells. Blood. 2014;**123**(18):2759-2767

[23] Ruiz FA, Lea CR, Oldfield E, Docampo R. Human platelet dense granules contain polyphosphate and are similar to acidocalcisomes of bacteria and unicellular eukaryotes. The Journal of Biological Chemistry. 2004;**279**(43):44250-44257

[24] Heijnen H, van der Sluijs P. Platelet secretory behaviour: as diverse as the granules…or not? Journal of Thrombosis and Haemostasis. 2015;**13**(12):2141-2151

[25] Nurden AT, Nurden P. The Gray Platelet syndrome: Clinical spectrum of the disease. Blood Reviews. 2007;**21**(1):21-36

[26] Zharikov S, Shiva S. Platelet mitochondrial function: From regulation of thrombosis to biomarker of disease. Biochemical Society Transactions. 2013;**41**(1):118-123

[27] Jobe SM, Wilson KM, Leo L, Raimondi A, Molkentin JD, Lentz SR, et al. Critical role for the mitochondrial permeability transition pore and cyclophilin D in platelet activation and thrombosis. Blood. 2008;**111**(3):1257-1265

[28] Choo HJ, Saafir TB, Mkumba L, Wagner MB, Jobe SM. Mitochondrial calcium and reactive oxygen species regulate agonist-initiated platelet phosphatidylserine exposure. Arteriosclerosis, Thrombosis, and Vascular Biology. 2012;**32**(12):2946-2955

[29] Hayashi T, Tanaka S, Hori Y, Hirayama F, Sato EF, Inoue M. Role of mitochondria in the maintenance of platelet function during in vitro storage. Transfusion Medicine (Oxford, England). 2011;**21**(3):166-174

[30] Boudreau LH, Duchez AC, Cloutier N, Soulet D, Martin N, Bollinger J, et al. Platelets release mitochondria serving as substrate for bactericidal group IIA-secreted phospholipase A2 to promote inflammation. Blood. 2014;**124**(14):2173-2183

[31] Hynes RO, Zhao Q. The evolution of cell adhesion. The Journal of Cell Biology. 2000;**150**(2):F89-F96

[32] Cosemans JM, Iserbyt BF, Deckmyn H, Heemskerk JW. Multiple ways to switch platelet integrins on and off. Journal of Thrombosis and Haemostasis. 2008;**6**(8):1253-1261

[33] Bennett JS, Berger BW, Billings PC. The structure and function of platelet integrins. Journal of Thrombosis and Haemostasis. 2009;**7**(Suppl 1):200-205

[34] Shattil SJ, Newman PJ. Integrins: Dynamic scaffolds for adhesion and signaling in platelets. Blood. 2004;**104**(6):1606-1615

[35] Humphries JD, Byron A, Humphries MJ. Integrin ligands at a glance. Journal of Cell Science. 2006;**119**(Pt 19):3901-3903

[36] Ohlmann P, de Castro S, Brown GGJ, Gachet C, Jacobson KA, Harden TK. Quantification of recombinant and platelet P2Y(1) receptors utilizing a [(125)I]-labeled high-affinity antagonist 2-iodo-N(6)-methyl-(N)-methanocarba-2′-deoxyadenosine-3′,5′-bisphosphate ([(125)I]MRS2500). Pharmacological Research. 2010;**62**(4):344-351

[37] Rivera J, Lozano ML, Navarro-Nunez L, Vicente V. Platelet receptors and signaling in the dynamics of thrombus formation. Haematologica. 2009;**94**(5):700-711

[38] Gale AJ. Continuing education course #2: Current understanding of hemostasis. Toxicologic Pathology. 2011;**39**(1):273-280

[39] Clemetson KJ. Platelets and primary haemostasis. Thrombosis Research. 2012;**129**(3): 220-224

[40] Ruggeri ZM, Mendolicchio GL. Adhesion mechanisms in platelet function. Circulation Research. 2007;**100**(12):1673-1685

[41] Broos K, Feys HB, De Meyer SF, Vanhoorelbeke K, Deckmyn H. Platelets at work in primary hemostasis. Blood Reviews. 2011;**25**(4):155-167

[42] Farndale RW, Siljander PR, Onley DJ, Sundaresan P, Knight CG, Barnes MJ. Collagen-platelet interactions: Recognition and signalling. Biochemical Society Symposium. 2003;**70**:81-94

[43] Cranmer SL, Ashworth KJ, Yao Y, Berndt MC, Ruggeri ZM, Andrews RK, et al. High shear-dependent loss of membrane integrity and defective platelet adhesion following disruption of the GPIbalpha-filamin interaction. Blood. 2011;**117**(9):2718-2727

[44] Li Z, Delaney MK, O'Brien KA, Du X. Signaling during platelet adhesion and activation. Arteriosclerosis, Thrombosis, and Vascular Biology. 2010;**30**(12):2341-2349

[45] Tsuji M, Ezumi Y, Arai M, Takayama H. A novel association of Fc receptor gamma-chain with glycoprotein VI and their co-expression as a collagen receptor in human platelets. The Journal of Biological Chemistry. 1997;**272**(38):23528-23531

[46] Jarvis GE, Atkinson BT, Snell DC, Watson SP. Distinct roles of GPVI and integrin alpha(2) beta(1) in platelet shape change and aggregation induced by different collagens. British Journal of Pharmacology. 2002;**137**(1):107-117

[47] Mazzucato M, Cozzi MR, Battiston M, Jandrot-Perrus M, Mongiat M, Marchese P, et al. Distinct spatio-temporal Ca^{2+} signaling elicited by integrin alpha2beta1 and glycoprotein VI under flow. Blood. 2009;**114**(13):2793-2801

[48] Kaplan ZS, Jackson SP. The role of platelets in atherothrombosis. Hematology American Society of Hematology Education Program. 2011;**2011**:51-61

[49] Andrews RK, Berndt MC. Platelet physiology and thrombosis. Thrombosis Research. 2004;**114**(5-6):447-453

[50] Nakahata N. Thromboxane A2: Physiology/pathophysiology, cellular signal transduction and pharmacology. Pharmacology & Therapeutics. 2008;**118**(1):18-35

[51] Angiolillo DJ, Ueno M, Goto S. Basic principles of platelet biology and clinical implications. Circulation Journal: Official Journal of the Japanese Circulation Society. 2010;**74**(4):597-607

[52] Monroe DM, Hoffman M, Roberts HR. Platelets and thrombin generation. Arteriosclerosis, Thrombosis, and Vascular Biology. 2002;**22**(9):1381-1389

[53] de Candia M, Fiorella F, Lopopolo G, Carotti A, Romano MR, Lograno MD, et al. Synthesis and biological evaluation of direct thrombin inhibitors bearing 4-(piperidin-1-yl)pyridine at the P1 position with potent anticoagulant activity. Journal of Medicinal Chemistry. 2013;**56**(21):8696-8711

[54] Horn NA, Anastase DM, Hecker KE, Baumert JH, Robitzsch T, Rossaint R. Epinephrine enhances platelet-neutrophil adhesion in whole blood in vitro. Anesthesia and Analgesia. 2005;**100**(2):520-526

[55] Andre P, Prasad KS, Denis CV, He M, Papalia JM, Hynes RO, et al. CD40L stabilizes arterial thrombi by a beta3 integrin-dependent mechanism. Nature Medicine. 2002;**8**(3):247-252

[56] Kulkarni S, Dopheide SM, Yap CL, Ravanat C, Freund M, Mangin P, et al. A revised model of platelet aggregation. The Journal of Clinical Investigation. 2000;**105**(6):783-791

[57] Ni H, Denis CV, Subbarao S, Degen JL, Sato TN, Hynes RO, et al. Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen. The Journal of Clinical Investigation. 2000;**106**(3):385-392

[58] Cho J, Mosher DF. Role of fibronectin assembly in platelet thrombus formation. Journal of Thrombosis and Haemostasis. 2006;**4**(7):1461-1469

[59] Dunne E, Spring CM, Reheman A, Jin W, Berndt MC, Newman DK, et al. Cadherin 6 has a functional role in platelet aggregation and thrombus formation. Arteriosclerosis, Thrombosis, and Vascular Biology. 2012;**32**(7):1724-1731

[60] Dai B, Wu P, Xue F, Yang R, Yu Z, Dai K, et al. Integrin-alphaIIbbeta3-mediated outside-in signalling activates a negative feedback pathway to suppress platelet activation. Thrombosis and Haemostasis. 2016;**116**(5):918-930

[61] Sakariassen KS, Orning L, Turitto VT. The impact of blood shear rate on arterial thrombus formation. Future Science OA. 2015;**1**(4):FSO30

[62] Jackson SP. The growing complexity of platelet aggregation. Blood. 2007;**109**(12):5087-5095

[63] Munnix IC, Cosemans JM, Auger JM, Heemskerk JW. Platelet response heterogeneity in thrombus formation. Thrombosis and Haemostasis. 2009;**102**(6):1149-1156

[64] Jones CI, Barrett NE, Moraes LA, Gibbins JM, Jackson DE. Endogenous inhibitory mechanisms and the regulation of platelet function. Methods in Molecular Biology (Clifton, NJ). 2012;**788**:341-366

[65] Steele BM, Harper MT, Macaulay IC, Morrell CN, Perez-Tamayo A, Foy M, et al. Canonical Wnt signaling negatively regulates platelet function. Proceedings of the National Academy of Sciences of the United States of America. 2009;**106**(47):19836-19841

[66] Macaulay IC, Thon JN, Tijssen MR, Steele BM, MacDonald BT, Meade G, et al. Canonical Wnt signaling in megakaryocytes regulates proplatelet formation. Blood. 2013;**121**(1): 188-196

[67] Bye A, Rosjo H, Aspenes ST, Condorelli G, Omland T, Wisloff U. Circulating microRNAs and aerobic fitness—The HUNT-study. PLoS One. 2013;**8**(2):e57496

[68] Smolenski A. Novel roles of cAMP/cGMP-dependent signaling in platelets. Journal of Thrombosis and Haemostasis. 2012;**10**(2):167-176

[69] Gkaliagkousi E, Ferro A. Nitric oxide signalling in the regulation of cardiovascular and platelet function. Frontiers in Bioscience (Landmark edition). 2011;**16**:1873-1897

[70] Gambaryan S, Tsikas D. A review and discussion of platelet nitric oxide and nitric oxide synthase: Do blood platelets produce nitric oxide from L-arginine or nitrite? Amino Acids. 2015;**47**(9):1779-1793

[71] Benz PM, Fleming I. Can erythrocytes release biologically active NO? Cell Communication and Signaling. 2016;**14**(1):22

[72] Cognasse F, Nguyen KA, Damien P, McNicol A, Pozzetto B, Hamzeh-Cognasse H, et al. The inflammatory role of platelets via their TLRs and Siglec receptors. Frontiers in Immunology. 2015;**6**:83

[73] Zago AC, Simon DI, Wang Y, Sakuma M, Chen Z, Croce K, et al. The importance of the interaction between leukocyte integrin mac-1 and platelet glycoprotein Ib-a for leukocyte recruitment by platelets and for the inflammatory response to vascular injury. Arquivos Brasileiros de Cardiologia. 2008;**90**(1):54-63

[74] Boilard E, Nigrovic PA, Larabee K, Watts GFM, Coblyn JS, Weinblatt ME, et al. Platelets amplify inflammation in arthritis via collagen-dependent microparticle production. Science. 2010;**327**(5965):580-583

[75] Metcalf Pate KA, Lyons CE, Dorsey JL, Queen SE, Adams RJ, Morrell CN, et al. TGFbeta-mediated downregulation of thrombopoietin is associated with platelet decline in

asymptomatic SIV infection. Journal of Acquired Immune Deficiency Syndromes (1999). 2014;**65**(5):510-516

[76] Guclu E, Durmaz Y, Karabay O. Effect of severe sepsis on platelet count and their indices. African Health Sciences. 2013;**13**(2):333-338

[77] Labelle M, Begum S, Hynes RO. Direct signaling between platelets and cancer cells induces an epithelial-mesenchymal-like transition and promotes metastasis. Cancer Cell. 2011;**20**(5):576-590

[78] Cooke NM, Spillane CD, Sheils O, O'Leary J, Kenny D. Aspirin and P2Y12 inhibition attenuate platelet-induced ovarian cancer cell invasion. BMC Cancer. 2015;**15**:627

[79] Erpenbeck L, Schon MP. Deadly allies: The fatal interplay between platelets and metastasizing cancer cells. Blood. 2010;**115**(17):3427-3436

[80] Kesikburun S, Tan AK, Yilmaz B, Yasar E, Yazicioglu K. Platelet-rich plasma injections in the treatment of chronic rotator cuff tendinopathy: A randomized controlled trial with 1-year follow-up. The American Journal of Sports Medicine. 2013;**41**(11):2609-2616

[81] Paniccia R, Priora R, Liotta AA, Abbate R. Platelet function tests: A comparative review. Vascular Health and Risk Management. 2015;**11**:133-148

[82] Duke WW. The relation of blood platelets to hemorrhagic disease. By W.W. Duke. Journal of the American Medical Association. 1983;**250**(9):1201-1209

[83] Born GV, Haslam RJ, Goldman M. Comparative effectiveness of adenosine analogues as inhibitors of blood-platelet aggregation and as vasodilators in man. Nature. 1965;**205**: 678-680

[84] Karger R, Reuter K, Rohlfs J, Nimsky C, Sure U, Kretschmer V. The platelet function analyzer (PFA-100) as a screening tool in neurosurgery. ISRN Hematology. 2012;**2012**:839242

[85] Varon D, Dardik R, Shenkman B, Kotev-Emeth S, Farzame N, Tamarin I, et al. A new method for quantitative analysis of whole blood platelet interaction with extracellular matrix under flow conditions. Thrombosis Research. 1997;**85**(4):283-294

[86] Peerschke EI, Silver RT, Weksler BB, Yin W, Bernhardt B, Varon D. Examination of platelet function in whole blood under dynamic flow conditions with the cone and plate(let) analyzer: Effect of erythrocytosis and thrombocytosis. American Journal of Clinical Pathology. 2007;**127**(3):422-428

[87] Savion N, Varon D. Impact—The cone and plate(let) analyzer: Testing platelet function and anti-platelet drug response. Pathophysiology of Haemostasis and Thrombosis. 2006;**35**(1-2):83-88

[88] Anand SX, Kim MC, Kamran M, Sharma SK, Kini AS, Fareed J, et al. Comparison of platelet function and morphology in patients undergoing percutaneous coronary intervention receiving bivalirudin versus unfractionated heparin versus clopidogrel pretreatment and bivalirudin. The American Journal of Cardiology. 2007;**100**(3):417-424

[89] Koshy SK, Salahuddin S, Karunakaran B, Nalakath SY, Bhaskaran J, Haridas PV, et al. Aspirin and clopidogrel resistance using the cone and plate(let) analyser in Indian patients with coronary artery disease. Heart Asia. 2014;**6**(1):159-162

[90] Goldschmidt N, Spectre G, Brill A, Zelig O, Goldfarb A, Rachmilewitz E, et al. Increased platelet adhesion under flow conditions is induced by both thalassemic platelets and red blood cells. Thrombosis and Haemostasis. 2008;**100**(5):864-870

[91] Revel-Vilk S, Varon D, Shai E, Agmon Y, Hyam E, Daas N, et al. Evaluation of children with a suspected bleeding disorder applying the impact-R [cone and plate(let) analyzer]. Journal of Thrombosis and Haemostasis. 2009;**7**(12):1990-1996

[92] Levy-Shraga Y, Maayan-Metzger A, Lubetsky A, Shenkman B, Kuint J, Martinowitz U, et al. Platelet function of newborns as tested by cone and plate(let) analyzer correlates with gestational age. Acta Haematologica. 2006;**115**(3-4):152-156

[93] Budak YU, Polat M, Huysal K. The use of platelet indices, plateletcrit, mean platelet volume and platelet distribution width in emergency non-traumatic abdominal surgery: A systematic review. Biochemia Medica. 2016;**26**(2):178-193

[94] Khandekar MM, Khurana AS, Deshmukh SD, Kakrani AL, Katdare AD, Inamdar AK. Platelet volume indices in patients with coronary artery disease and acute myocardial infarction: An Indian scenario. Journal of Clinical Pathology. 2006;**59**(2):146-149

[95] Madjid M, Fatemi O. Components of the complete blood count as risk predictors for coronary heart disease: In-depth review and update. Texas Heart Institute Journal. 2013;**40**(1):17-29

[96] Vagdatli E, Gounari E, Lazaridou E, Katsibourlia E, Tsikopoulou F, Labrianou I. Platelet distribution width: A simple, practical and specific marker of activation of coagulation. Hippokratia. 2010;**14**(1):28-32

[97] Mangalpally KK, Siqueiros-Garcia A, Vaduganathan M, Dong JF, Kleiman NS, Guthikonda S. Platelet activation patterns in platelet size sub-populations: Differential responses to aspirin in vitro. Journal of Thrombosis and Thrombolysis. 2010;**30**(3):251-262

[98] Yilmaz T, Yilmaz A. Relationship between altered platelet morphological parameters and retinopathy in patients with type 2 diabetes mellitus. Journal of Ophthalmology. 2016;**2016**:9213623

[99] Yilmaz T, Yilmaz A. Altered platelet morphological parameters in patients with retinal vein occlusion. European Review for Medical and Pharmacological Sciences. 2016;**20**(10):1934-1939

[100] Leite NR, Siqueira de Medeiros M, Mury WV, Matsuura C, Perszel MB, Noronha Filho G, et al. Platelet hyperaggregability in obesity: Is there a role for nitric oxide impairment and oxidative stress? Clinical and Experimental Pharmacology & Physiology. 2016;**43**(8):738-744

[101] Rechcinski T, Jasinska A, Forys J, Krzeminska-Pakula M, Wierzbowska-Drabik K, Plewka M, et al. Prognostic value of platelet indices after acute myocardial infarction treated with primary percutaneous coronary intervention. Cardiology Journal. 2013;**20**(5):491-498

[102] Gul M, Uyarel H, Akgul O, Akkaya E, Surgit O, Cakmak HA, et al. Long-term prognostic significance of admission plateletcrit values in patients with non-ST elevation myocardial infarction. Blood Coagulation & Fibrinolysis. 2016;**27**(6):696-701

[103] Ho CH. White blood cell and platelet counts could affect whole blood viscosity. Journal of the Chinese Medical Association. 2004;**67**(8):394-397

[104] Cetin M, Bakirci EM, Baysal E, Tasolar H, Balli M, Cakici M, et al. Increased platelet distribution width is associated with ST-segment elevation myocardial infarction and thrombolysis failure. Angiology. 2014;**65**(8):737-743

[105] Chesnutt JK, Han HC. Effect of red blood cells on platelet activation and thrombus formation in tortuous arterioles. Frontiers in Bioengineering and Biotechnology. 2013;**1**:18

[106] McRedmond JP, Park SD, Reilly DF, Coppinger JA, Maguire PB, Shields DC, et al. Integration of proteomics and genomics in platelets: A profile of platelet proteins and platelet-specific genes. Molecular & Cellular Proteomics. 2004;**3**(2):133-144

[107] Ferroni P, Riondino S, Vazzana N, Santoro N, Guadagni F, Davi G. Biomarkers of platelet activation in acute coronary syndromes. Thrombosis and Haemostasis. 2012;**108**(6):1109-1123

[108] Baccarelli AA, Byun HM. Platelet mitochondrial DNA methylation: A potential new marker of cardiovascular disease. Clinical Epigenetics. 2015;**7**:44

[109] Kaudewitz D, Skroblin P, Bender LH, Barwari T, Willeit P, Pechlaner R, et al. Association of MicroRNAs and YRNAs with platelet function. Circulation Research. 2016;**118**(3):420-432

[110] Dahiya N, Sarachana T, Kulkarni S, Wood WHIII, Zhang Y, Becker KG, et al. miR-570 interacts with mitochondrial ATPase subunit g (ATP5L) encoding mRNA in stored platelets. Platelets. 2017;**28**(1):74-81

Chapter 6

Platelets: Functional Biomarkers of Epigenetic Drift

Laura Twomey, Robert G. Wallace, Marco Mangone,
Bernard Degryse, Sinead Sheridan,
Michael Harrison, Niall Moyna,
Gerardene Meade-Murphy, Nastassia Navasiolava,
Marc-Antoine Custaud and Ronan P. Murphy

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.83447

Abstract

Cardiovascular disease (CVD) risk factors can be classed as modifiable or non-modifiable. Physical inactivity and obesity represent major behavioural risk factors for the initiation, development and progression of CVD. Platelet dysfunction is pivotal to the aetiology of CVD, a chronic vascular inflammatory condition, which is characterised by a lag time between onset and clinical manifestation. This indicates the role of epigenetic drift, defined by stochastic patterns of gene expression not dependent on dynamic changes in coding DNA. The epigenome, a collection of chemical marks on DNA and histones, is established during embryogenesis and modified by age and lifestyle. Biogenesis and effector function of non-coding RNA, such as microRNA, play a regulatory role in gene expression and thus the epigenetic mechanism. In this chapter, we will focus on the effect of the modifiable risk factors of physical activity/inactivity and overweight/obesity on platelet function, via epigenetic changes in both *megakaryocytopoiesis* and *thrombopoiesis*. We will also discuss the role of acute exercise on platelet function and the impact of cardiorespiratory fitness (CRF) on platelet responses to acute exercise. This chapter will highlight the potential role of platelets as circulating functional biomarkers of epigenetic drift to implement, optimise and monitor CVD preventive management strategies.

Keywords: platelets, epigenetics, microRNA, lifestyle, physical activity, physical inactivity, cardiovascular disease, preventive medicine

1. Introduction

1.1. Epigenetics

Epigenetics describes modifiable changes that occur to genes, *via* chemical modifications and/or varying states of chromatin organisation and structure, which alter the gene expression without altering the DNA sequence itself. Smoking habits, obesity, ageing and physical fitness among others are examples of environmental factors that have been suggested to have a long-term influence on epigenetic changes [1]. Epigenetics may be classed as three distinct but highly interconnected processes; DNA methylation, histone modification and RNA-associated silencing (**Figure 1**). DNA methylation and histone modification alter DNA accessibility for transcriptional machinery and chromatin structure. These changes are heritable and can be passed down between generations through either mitosis or meiosis. DNA methylation involves the addition of a methyl group to the 5-position of cytosine by DNA methyl-transferases, at areas known as CpG islands. Methyl groups control gene expression by binding to promoter sites of the gene. This changes the affinity of methylation-sensitive binding proteins, and is associated with transcriptional gene silencing [2]. Whilst required for normal development, changes in DNA methylation have been linked to CVD conditions such as atherosclerosis. For example, the athero-protective oestrogen receptor genes ESR1 and ESR2, usually expressed in SMCs, are hyper-methylated in atherosclerosis [3, 4].

Unlike the platelet transcriptome and proteome, the investigation of epigenetic processes is an almost completely unexplored area in platelet biology, as analysis of these mechanisms requires DNA [2]. Although anucleate, platelets have functionally active mitochondria, with mitochondrial DNA (mtDNA) that can also be methylated, moderating the control of mitochondrial gene expression. Interestingly, Zhong and colleagues recently reported that *de novo* DNA synthesis in mitochondria, and its subsequent oxidation, plays a key role in triggering the innate immune response. Mitochondria can regulate how immune cells respond to infection and tissue damage, producing pro- or anti-inflammatory signals by regulating Krebs cycle metabolites or the production of reactive oxygen species (ROS). More and more examples are being found of mitochondrial functions being repurposed in unexpected ways to contribute to many biological processes, including inflammatory signalling [5].

Understanding epigenetic regulation of mitochondrial genes in platelets is proving crucial to understanding their implication in CVD development. Novel research by Baccarelli and Byun showed that CVD patients had significantly higher platelet mtDNA methylation than healthy individuals in MT-CO1, MT-C02, MT-CO3 and MT-TL1 genes involved in ATP synthesis [6]. These results suggest that DNA methylation in platelet mitochondria could be a potential contributor to CVD development through the regulation of platelet function.

Histone, proteins that structure DNA into units known as nucleosomes, can be modified at their amino-acid tails. Histone modifications refer to the post-translational alterations of the N-termini of these tails that subsequently modify histone-DNA interactions [7]. Acetylation is a major type of histone modification involving the addition or removal of an acetyl group. This process is catalysed by proteins known as histone acetyltransferases (HATs) and histone

http://dx.doi.org/10.5772/intechopen.83447

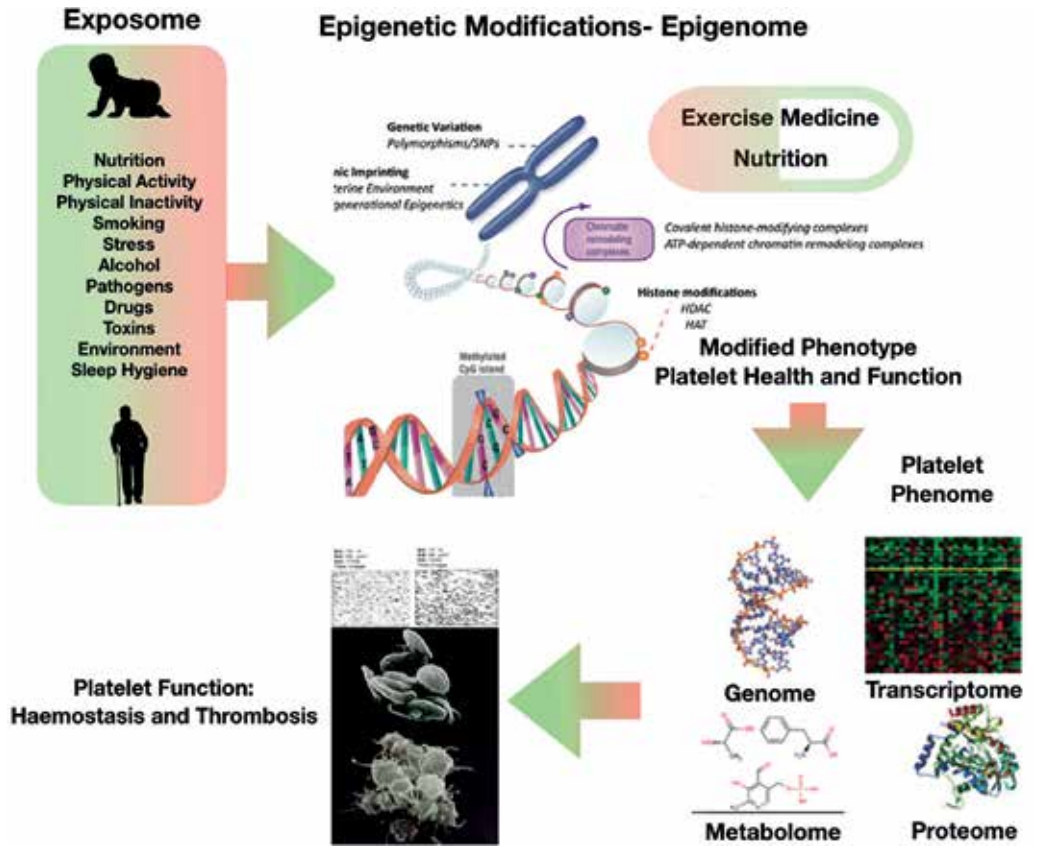

Figure 1. Modifiable risk factors covalently alter the static genome by processes involving epigenetic writers, erasers and readers, which fine-tune gene expression in an age and lifestyle dependent manner, a process known as *epigenetic drift*. The term epigenetics refers to stable patterns of gene expression and they are not dependent on dynamic changes in coding DNA. These gene expression states are encoded in the epigenome—a collection of chemical marks on DNA or on histones that are established during embryogenesis and are modified by age and environment over a person's lifetime. Studies have uncovered stochastic DNA methylation drift that reflects imperfect maintenance of epigenetic marks. We hypothesise that *drift* creates epigenetic mosaicism in ageing haemopoietic stem cells in the bone marrow. This in turn impacts on HSC differentiation, maturation and platelet production. This may highlight platelets as a functional diagnostic index of cardiovascular competence. The fact that the initiation and progression of CVD is characterised by a lag time between onset and clinical manifestation provides a window of opportunity for the implementation of intervention strategies to reduce the CVD burden. In addition, such studies will better inform primary, secondary and tertiary preventive strategies—promoting an ageing well paradigm and optimising a person's disease free years.

deacetyltransferases (HDACs). This mechanism alters chromatin structure (heterochromatin versus euchromatin) to influence gene expression [3].

RNA-based epigenetic processes involve non-coding RNA (ncRNA) and are factors in the chromatin-based regulation of gene expression [8]. ncRNAs can be classified as either long or short. Whilst long ncRNAs are a major form of RNA-based epigenetic regulation, some small ncRNAs also have a function in chromatin-based silencing. For example, microRNA (miRNA) is a subset of ncRNA that negatively regulates gene transcription by degrading

or repressing target mRNA [9]. miRNA can control the expression of important epigenetic regulators such as histone deacetylases and DNA methyl-transferases and similarly, DNA methylation and histone modification can control the expression of some miRNA, thereby forming a feedback loop [10]. This complex crosstalk between miRNA and epigenetic pathways forms an epigenetic-miRNA regulatory circuit, arranging the whole gene expression profile. Disruption of this circuit interferes with normal physiological functions and can contribute to disease process.

Individuals age differently and lifestyle factors such as exercise or smoking have been shown to delay or accelerate the ageing process, respectively [11]. These observations have resulted in the search for molecular markers to predict and monitor age-associated disease. DNA methylation is associated with chronological age over time [12]. *Epigenetic drift* is the term given to epigenetic modifications as they occur as a direct consequence of age [13]. This was previously observed when DNA methylation marks in identical twins differed increasingly as a function of age [1]. Monozygous twins share a common genotype and while this study found that the twins were epigenetically synonymous during childhood, older twins showed significant differences in their total content and dispersal of histone acetylation and DNA methylation. Disparity in these epigenetic marks between twins may be as a result of lifestyle influences such as diet, physical activity levels, stress and smoking.

Epigenetic drift affects the majority of the genome over time leading to biological ageing (**Figure 2**). Ageing is a natural process associated with the de-regulation of histone tags, senescence-associated lncRNA, a gradual de-regulation of DNA methylation, in a potential linear fashion depicted by age-predictive linear models [14]. However, an individual exposed to either environmental or genetic risk factors may show signs of premature ageing as a result of either lifestyle or environmental risk factors. De-regulation of DNA methylation can increase the susceptibility to chronic diseases like CVD. Furthermore, it has been hypothesised that a healthy lifestyle may reserve a more intact epigenome, promoting longevity [15]. In a recent compelling study by Horvath and colleagues, a novel, sensitive and highly robust DNAm age estimator (based on 391 CpGs) was developed for human fibroblasts, keratinocytes, buccal cells, endothelial cells, lymphoblastoid cells, skin, blood and saliva samples [16]. This seminal research builds upon and overcomes the limitations of the two ground-breaking studies on the epigenetic clock (biological/molecular age) and its relationship with chronological age. These studies, one a blood-based age estimator [14] and the other a pan-tissue estimator [17], facilitated age estimates (DNAmAge) that are widely used in epidemiological studies. The novel 'skin and blood clock' overcomes the technical and sensitivity limitations of the previous DNAmAge biomarker panels.

DNA methylation undergoes extensive changes during differentiation of self-renewing stem cells [18, 19]. Indeed, DNA methylation is involved in the production of MKs and subsequent transcription. Lifestyle components such as physical inactivity and obesity may incur epigenetic changes in the production of platelets from megakaryocytes. Thus, platelets could signify a marker of megakaryocyte epigenetic drift, holding substantial predictive potential of disease. Epigenetic changes in the megakaryocyte genome such as hypomethylation of genes

http://dx.doi.org/10.5772/intechopen.83447

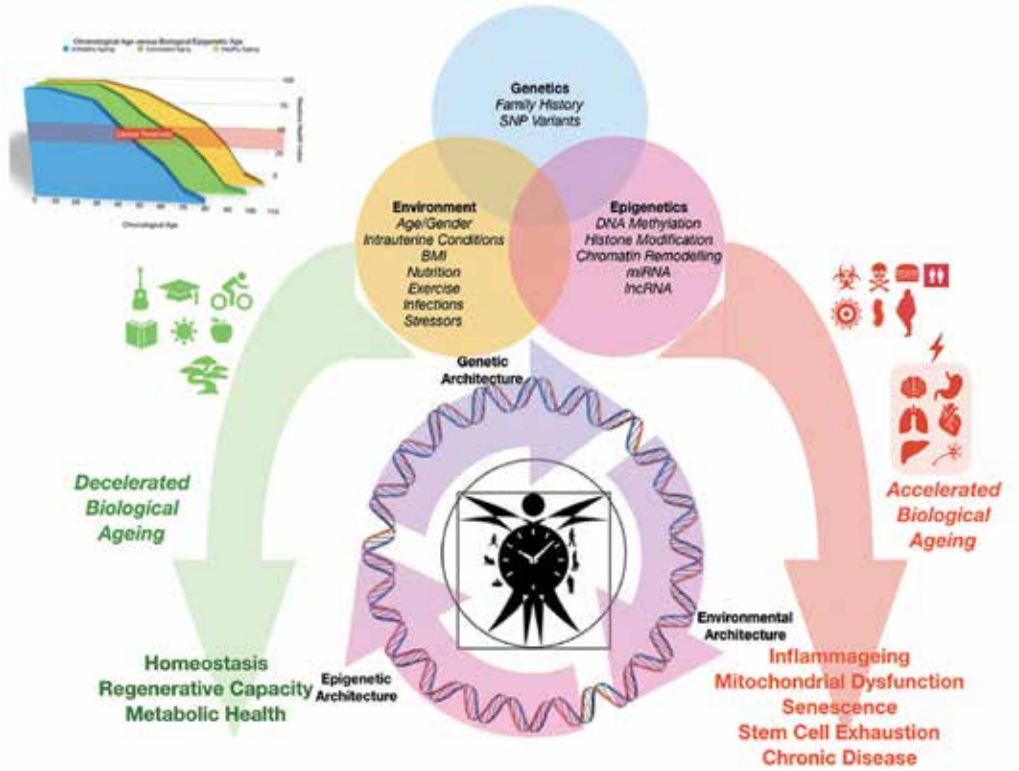

Figure 2. Schematic representation of ageing well versus accelerated ageing. Poor lifestyle choices such as inactivity and diet are rapidly becoming a global pandemic, accelerating many chronic illnesses such as cardiovascular disease, metabolic disorder, diabetes, Alzheimer's disease and cancer. Understanding their pathophysiology is important for the development of future therapeutic interventions, stratification of clinical trials and to challenge our current perception of engaging with cost effective measures such as lifestyle management. The aetiology of age-related chronic illnesses involves a complex interplay between many biological processes and is modulated by non-modifiable and modifiable risk factors.

determining PLT or changes in histone acetylation with aging have been suggested to play an important role in platelet function [20].

miRNAs are short [18–24] nucleotide long, non-coding RNAs that function in post-transcriptional regulation of gene expression. They inhibit translation by binding with the 3′-untranslated (UTR) regions of their target mRNA. Here, the miRNA promotes silencing of various genes [21, 22], hence now termed 'fine-tuners' of cellular phenotypes. They are thought to be involved in the regulation of ~60% of human genes [23, 24]. miRNA can be classed as intronic, exonic and intergenic miRNA, according to the location of their encoding genes [25]. Intronic miRNAs account for approximately 70% of all transcribed miRNA [26, 27]. Intergenic miRNAs are found between two protein-coding genes and employ their own promoters and regulatory molecules. As miRNAs target mRNA by imperfect binding, each miRNA has multiple targets, enabling miRNAs to regulate over half of the human genome [28]. The miRNA population within a cell can be highly concentrated, with tens of thousands of miRNA copies per cell.

They possess a long half-life (a half-life of between 28 and 220 h has been reported) and are very stable [29]. Turnover of mature miRNA is required for rapid changes in miRNA expression profiles. Regulation of miRNA maturation occurs during various steps throughout their biogenesis at both a transcriptional and post-transcriptional level [30]. Transcriptional regulation involves alterations to the expression of a host gene such as epigenetic regulation (where miRNA genes located near CpG islands in the genome are found to be hyper-methylated). Post-transcriptional mechanisms define modifications in miRNA processing and stability [31].

2. Platelet Epigenetics

2.1. Platelet miRNA

Platelet function is a highly regulated process. Despite their anucleate nature, platelets accommodate a small but competent transcriptome that is employed for translation of various proteins with significant physiological functions. Platelets have been shown to retain genetic material derived from their megakaryocyte precursor. Approximately 32% of all human genes are present in platelets at the mRNA level [23]. It is well accepted that platelets contain the necessary splicing machinery, rough ER and polyribosomes that allow the synthesis of proteins required for their functioning [32]. Perhaps due to the requirement of sustaining a proteome over an ~8-day life span, the fact that the average half-life of a cellular protein is 46 h, or the necessity to adapt to environmental stimuli, it is equitable to assume that the platelet must also retain its transcriptome, as well as processes of nucleated cells such as splicing, translating and post-transcriptional RNA mechanisms. The fact that platelets contain mRNA and are capable of protein synthesis has raised the issue of how these mRNAs are regulated. Notably, stored platelets in blood banks can synthesis integrin β3 [33]. The existence and functionality of a miRNA pathway in the anucleate human platelet was first described in a landmark study by Landry and co-workers [34], who showed by locked nucleic acid (LNA) microarray profiling, that platelets harboured an impressive number (219) of miRNA. Further analysis discovered the presence of functional processing miRNA machinery in platelets—Dicer and Ago2—suggesting that partial biogenesis of mature miRNA from pre-miRNA could occur within platelets themselves [34, 35] as pre-miRNAs have been identified at low levels (21 transcripts) in platelets [23]. Star or passenger strand miRNA have also been identified [36]. Accordingly, the detection of nuclear miRNA microprocessor Drosha and DGCR8 in platelets has not been observed, consistent with their anucleate nature. Moreover, miRNA-associated Ago2 complexes were identified, in addition to the presence of $P2Y_{12}$ in Ago2 precipitates, suggesting a regulation of $P2Y_{12}$ by miRNAs [34].

The next breakthrough study in platelet miRNA biology revealed that a protein involved in platelet granule release, platelet vesicle-associated membrane protein 8 (VAMP8), was associated with distinctly different platelet aggregation responses to epinephrine in healthy donors, and that VAMP8 was regulated by miR-86 [37]. Since then, the platelet miRNA field has grown exponentially, whereby a number of studies have suggested a physiological role

for miRNA in the regulation of platelet function. Most notably, research by Nagalla et al. [38], who focused on the roles of miRNA as biomarkers of platelet reactivity and controllers of platelet mRNA disparity, demonstrated that miRNA profiles of healthy subjects (n = 19) were associated with the response of platelet aggregation to epinephrine [38]. They also employed a computational approach to produce possible miRNA-mRNA pairs (miR-200b: PRKAR2B, miR-495: KLHL5 and miR-107: CLOCK), pairings which were experimentally validated in cell lines. Networks of miRNA-mRNA pairs also associated with age, gender and race [39, 40]. Other reports on agonist-induced platelet activation by thrombin (and ADP) show differential expression of platelet miRNA compared to resting platelets [41].

Progression in miRNA detection techniques has led to the revelation of 40 new miRNA sequences, expanding the total amount of platelet expressed miRNAs to more than twice that (544) of the initial finding [23, 35, 42]. Transcriptomic approaches show that miRNA make up the majority (80%) of all small RNAs in platelets. Furthermore, comparison of RNA and miRNA by cell type showed that despite low RNA yields, platelets express high quantities of miRNA compared to their nucleated counterparts.

A number of highly expressed miRNA have been characterised in human platelets, some of which are involved in myeloid cell differentiation, megakaryocytopoiesis and thrombopoiesis. miR-223 has been identified as the most highly expressed platelet miRNA [38, 43, 44] and has roles in thrombopoiesis and megakaryocyte differentiation [24]. miR-223 regulates ADP $P2Y_{12}$, a target for existing anti-platelet drug therapy. The 3′-UTR of $P2Y_{12}$ mRNA has been identified as complementary to the miR-223 seed region. Platelet miR-223 has also been observed to be decreased in subjects who show high levels of platelet activation whilst on clopidogrel therapy. Furthermore, miR-223-deficient mice show reduced bleeding times, larger thrombi and elevated sensitivity to low doses of thrombin, suggesting an important role of miR-223 in modulating platelet function [45]. miR-126 plays central roles in vascular inflammation and is thought to be the second most highly expressed miRNA in platelets [46]. miR-126 was found to correlate with circulating P-Selectin levels in T2DM subjects and this level was sensitive to aspirin treatment, signifying a platelet origin. miR-126 is postulated to regulate ADAM9 and $P2Y_{12}$ receptor expression in platelets and inhibition of miR-126 in mice distinctly reduces platelet aggregation [47].

Existence of miRNA in platelets is multifaceted. Besides their obvious function as regulators of platelet protein expression, platelet miRNAs have been labelled as biomarkers of disease and platelet activation, markers of mature megakaryocyte miRNA and as a means of understanding megakaryocyte/platelet gene expression [48]. The majority of platelet miRNA are supposedly formed in the megakaryocyte and packaged into platelets upon formation. For example, miR-146b positively regulates megakaryopoieses by targeting and down regulating the megakaryopoieses inhibitor PDGFRA [49, 50]. miR-142 has also been reported to inhibit megakaryocyte production. In miR-142 knockout mice, platelet counts are decreased and MK differentiation is modified, including reduced proplatelet network establishment [51]. The total extent to which MK and platelet mature miRNA patterns correlate remains an area of active investigation. A significant correlation between the miRNA levels was found using three separate studies [34, 52, 53].

Perhaps, the most intriguing feature regarding platelet miRNA is their extracellular function. miRNA can be packaged and delivered to distant cells in the form of platelet microvesicles (PMV) and/or microparticles (PMP), fulfilling novel processes of gene regulation in target cells [54]. Initial studies by Laffont and Gidlöf demonstrated the functionality of platelet miRNA [24, 55]. Functional complexes of miR-223 and Argonaute 2 protein (Ago2) packaged in MVs from activated platelets were found to modulate the expression of targeted endothelial cell endogenous mRNA transcripts FBXW7 and EFNA1. This miR-223/Ago2 complex has also been shown to reduce expression levels of insulin-like growth factor 1 receptor in endothelial cells, and to promote human umbilical vein endothelial cell (HUVEC) apoptosis [56].

Gidlof et al. suggested that platelet miRNA could modulate vascular endothelial inflammatory responses [55]. They described a down regulation of intercellular adhesion molecule 1 (ICAM-1) gene expression in cultured human microvascular endothelial cells after exposure to miR-320b, which is secreted upon platelet activation and reduced in platelet thrombi aspirated from patients with ST-segment elevation myocardial infarction (STEMI). The relevance of this intercellular transfer was further reinforced when Liang et al., showed that platelet-released miR-223 through platelet MPs can encourage lung cancer cell invasion by targeting the tumour suppressor EPB41L3 [57].

Novel research has shown that platelet MPs containing miRNA can also be internalised by primary human macrophages and deliver functional miR-126-3p. miR-126-3p caused a down-regulation in the expression of four predicted mRNA targets of miR-126-3p and a reduction in macrophage cytokine release. This suggests that platelet miRNA-containing MPs can modify the macrophage transcriptome and potentially reprogram their function [58]. Finally, platelet-derived exosomes have recently been shown to carry miR-223, miR-339 and miR-21, which can be transferred to SMCs affecting PDGRFβ [13].

2.2. Platelets, lifestyle and miRNA in the aetiology of CVD

Efforts in coping with CVD require further understanding of its aetiology in order to develop effective management strategies. Epidemiological studies in adults have acknowledged a set of characteristic risk factors that predict the probability of a person developing clinical manifestations of disease [59, 60]. CVD risk factors are classed as modifiable or non-modifiable. Non-modifiable risk factors include age, ethnicity, gender and family history. Modifiable risk factors include hypertension, smoking, diabetes, unhealthy diet, cholesterol, physical inactivity (PI, sedentary lifestyle and low cardiorespiratory fitness) and overweight/obesity. Risk factors for CVD track from childhood into adulthood [61] and are strong predictors of subclinical atherosclerosis in early adulthood. The majority of CVD is caused by modifiable risk factors and up to 80% of CVD may be prevented if risk factors are avoided [62]. Physical inactivity and obesity are primary potent risk factors, both of which can severely impact platelet physiology.

Platelets have central roles in CVD [63] contributing to both early stages of endothelial dysfunction and advanced stages of the plaque rupture [64]. Platelets participate in early stage disease initiation through multiple mechanisms enabling adhesion to dysfunctional endothelium. Activated platelets express high levels of adhesion receptors (e.g., ICAM1, P-Selectin, CD40L) associated with oxidised-LDL (ox-LDL) that contributes to vascular inflammation

[65]. TLR signalling may also play a role in the progression of atherosclerosis by binding of lipopolysaccharides (LPS) to TLR4 on platelets and also mediating platelet-neutrophil interactions.

Direct cell-cell communication through platelet P-Selectin and CD40 ligand (CD40L) encourages inflammatory processes [66]. CD40L is thought to be at the heart of the atherosclerotic process, with 90% of circulating CD40L residing in platelets. CD40L is sent to the platelet surface upon activation, where it can initiate numerous inflammatory processes. The release of CD40L is intrinsically linked to αIIbβ3 as αIIbβ3 antagonists can block the release of sCD40L from activated platelets *in vitro*. Recently, platelet CD40 was shown to mediate the formation of platelet-leukocyte aggregates (stimulates leukocyte activation) and release inflammatory chemokines that activate endothelial cells, supporting atherosclerosis [66]. The significance of P-Selectin in atherosclerosis has been demonstrated in P-Selectin deficient animals that were protected from the disease. The role of platelet P-Selectin was clarified further by Huo et al., who illustrated that the introduction of P-Selectin expressing platelets into ApoE (−/−) mice accelerated atherosclerosis, whereas mice injected with platelets lacking P-Selectin formed smaller plaques [67].

Platelet-derived microparticles released upon activation may further amplify the progression of atherosclerosis through processes of adhesion, coagulation, inflammation and lipid metabolism [68]. Platelets also provide a huge repertoire of additional inflammatory mediators including a vast array of chemokines and cytokines that contribute to the crosstalk of platelets with other inflammatory cells—e.g., endothelial cells, monocytes, neutrophils, dendritic cells and T-cells [66]. The major function of platelets in atherosclerosis is the recruitment of leukocytes through direct receptor-ligand interactions or amplification of leukocyte recruitment through chemokine release. This bidirectional relationship is extremely important as platelets encourage leukocyte differentiation into a pro-adhesive and pro-migratory phenotype, and the leukocytes secrete mediators that reciprocally activate platelets.

Following atherosclerotic plaque rupture in severe CVD states, the exposure of thrombogenic substrates to circulating platelets instantly triggers platelet adhesion, activation and aggregation, forming a prothrombotic surface and subsequently encouraging thrombosis, vasoconstriction and vascular occlusion. Activated platelets expose phospholipids on their surface, which also promotes the coagulation cascade and subsequent fibrin production [64]. Given the critical roles of platelets in the pathogenesis of atherosclerosis and the development of acute thrombotic events, anti-platelet therapy has been widely employed in the primary and secondary prevention of CVD. Some of the current anti-platelet therapy drugs include Aspirin, which irreversibly inhibits cyclooxygenase to subsequently decrease TxA_2 production and limit platelet aggregation. Clopidogrel and Prasugrel are examples of $P2Y_{12}$ receptor antagonists that inhibit the soluble agonist ADP, whilst Tirofiban and Abciximab block αIIbβ3-ligand interactions. Other anti-platelet therapies include thrombin and phosphodiesterase inhibitors (block degradation of cyclic nucleotides) [69, 70].

Given the impact of miRNA gene regulation, it is unsurprising that the dysregulation of miRNA is implicated in CVD. miRNAs are central players in modulating gene expression of cells/platelets collectively involved in CVD, and mediate inflammation, lipid uptake and cell differentiation

in atherosclerosis. Platelet miRNA signatures (miR-25-3p, miR-221-3p and miR-374b-5) alter between patients with ST-segment elevation myocardial infarction (STEMI) and those with non-STEMI [71] suggesting that levels of platelet miRNA could impact platelet thrombogenicity and type of infarction. Furthermore, circulating miRNAs associated with the risk of MI (miR-126, miR-150, miR-223 and miR-197) are abundantly expressed in platelets. Platelet miRNA are implicated in premature CAD as two miRNAs in platelets are up-regulated in patients compared to controls (miR-340* and miR-624*), although whether or not they are the cause or consequence is currently unknown [72]. Besides their roles as mediators and biomarkers of CVD, platelet miRNA act as novel surrogate measures of the responsiveness to anti-platelet therapies used in CVD [73]. miR-223 levels are significantly down regulated in low responders to anti-platelet therapy [45, 74]. Furthermore, expression of platelet miR-26a has been linked with clopidogrel resistance during coronary stenting [75]. This theory is strengthened by research demonstrating how the switch from dual anti-platelet treatment with clopidogrel to ticagrelor is linked with significant changes in the level of platelet-specific circulating miRNAs, namely miR-223, miR-126 and miR-150 and miR-96 [76]. Other research investigating the effects of anti-platelet therapy on platelet miRNA levels showed that *in vitro* platelet activation resulted in transfer of miR-126 from platelets to plasma, whereas in aspirin-treated platelets, this process was not observed. *In vivo*, aspirin intake resulted in platelet inhibition and lower circulating platelet-derived miR-126 levels than were seen in untreated subjects [77]. Greater understanding of the meaning of platelet miRNA in CVD patients could aid in the diagnosis and treatment of these diseases.

2.3. Effect of obesity on platelet function

Obesity is a multifactorial condition involving a plethora of interrelated processes such as alterations in lipid metabolism, insulin resistance, inflammation, endothelial dysfunction, adipokine imbalance and oxidative stress. These metabolic aberrations have been postulated to be involved in platelet hyper-aggregability. Indeed, platelet activation markers are described as elevated in obesity, contributing to the inflammatory and prothrombotic state [78]. Subjects with overweight and obesity display increased platelet activation markers urinary-11-dehydro-TXB2 [79], MPV [80] and PLT [81]. Greater platelet activation (P-Selectin and PMP) is also linked to central arterial stiffness and carotid wall thickness amongst other atherosclerotic risk factors in overweight and obese subjects [82, 83]. The major mechanisms behind platelet function in obesity include a reduced sensitivity to insulin and resistance to their main inhibitory mediators PGI_2 and NO, elevated oxidative stress and an altered intracellular environment with increased cytosolic Ca^{2+} [84]. Platelets have insulin receptors which impact platelet function by regulating platelet response and sensitisation of platelets to inhibitory mechanism of PGI_2 and NO. In obese subjects, the anti-aggregating effect of insulin is diminished [85, 86].

Elevated oxidative stress also plays important roles in obesity-related platelet dysfunction. Oxidative stress results from an imbalance between the generation of free radicals and antioxidant enzymes [87]. High reactive oxygen species (ROS) generation by excess adipose tissue reduces NO bioavailability, enhancing surface expression of adhesion molecules, and enabling platelet activation and adhesion. Increased ROS also converts arachidonic acid into F_2-isoprostanes such as 8-iso-$PGF_{2\alpha}$ that can modulate platelet adhesive function [88]. Activated platelets also produce ROS [89], amplifying their own aggregatory potential by increasing

αIIbβ3 and CD40L expression [90, 91] and stimulating intraplatelet F_2-isoprostanes production. Both decreased NO synthesis and bioavailability from ECs and platelets contribute to the pathogenesis of obesity, likely promoting thrombosis. Research by Leite et al. describes a decrease of nitric oxide synthase (NOS) activity and cGMP levels with simultaneous platelet hyper-aggregability in obese subjects compared to healthy controls with impaired antioxidant responses as potential contributors [92]. Anfossi et al. showed that platelet sensitivity to anti-aggregatory effects of PGI_2 and NO is reduced in obesity [84]. Importantly, weight loss in obese subjects marks a reduction in platelet activation markers and can potentially reverse the platelet responsiveness to NO and prostacyclin [93, 94]. A 10% weight reduction in obese subjects resulted in significant reductions in BMI, endothelial dysfunction and platelet aggregation. The changes in platelet function were associated with improvement in insulin sensitivity, indicating a tight relationship between the two. Weight loss also resulted in reduction in lipid peroxidation markers [95] and P-Selectin expression in overweight CAD patients [96].

Although an association between obesity and platelet activation is evident, the molecular mechanisms responsible have only begun to surface [97]. Platelet RNA is reflective of pathological disease states where inflammatory transcript profiles (e.g., INFG, IL1R1, IL6 and TLR2) correlate significantly with increasing BMI [98], supporting the hypothesis that surplus fat could unfavourably alter the inflammatory potential of platelets. However, obesity can also cause dysregulation of other factors that control haemostasis such as microRNA (miRNA). There is increasing evidence to show that miRNA is involved in the pathogenesis of obesity [99], where plasma levels of miR-223 are reduced in obese compared to lean subjects, suggesting that the miR-223/$P2Y_{12}$ alliance could signify a contributing mechanism of platelet activation in obesity [36].

2.4. Role of physical activity on platelet function

Those who engage in regular physical activity or exercise have a reduced prevalence of CVD. PA has been extensively studied due to its beneficial effects on all-cause mortality. Evidence to support the inverse relationship between PA and either CVD, cancer or depression continues to accumulate. With regard to CVD, regular PA/exercise reduces blood pressure, serum triglycerides, total body fat and visceral fat and LDL cholesterol [100]. Differences in these known factors have been demonstrated to explain a large proportion of the inverse relationship between physical activity and CVD risk [101, 102]. However, over 40% of the inverse association remains unexplained. Although the beneficial effects of regular exercise on blood lipids and blood pressure have been well documented, research focusing on platelet function has only recently gained greater attention. Since platelets play a key role in the pathogenesis of CVD, the protective effect of exercise against CVD may be partially due to alterations of platelet function [103].

Aerobic fitness is measured by maximal oxygen uptake (VO_2 max) during incremental exercise and is globally acknowledged as the best assessment of cardiovascular fitness [104]. VO_2 max represents the maximal amount of oxygen that an individual can take in and use to produce energy. VO_2 max is a function of the ability of the cardiovascular system to deliver blood and oxygen to skeletal muscle, and the ability of skeletal muscle to extract this oxygen and use it to produce energy. Exercise effects on platelet function in both diseased and healthy

populations have elicited profound interest in the last decade. The majority of research surrounding platelet function and physical activity/exercise has focused on acute (single bout) aerobic exercise. Potential effects of acute exercise on platelet function (mainly aggregation) have been investigated through various studies in adult subjects with varying intra- and inter-individual results, making interpretation problematic. Differences in population type (e.g., CVD versus healthy), methods employed to assess platelet function and techniques to examine reactivity are the main reasons for discrepancies and lack of consistency between research groups [105]. Different platelet adhesion experimental protocols have provided no definitive consensus on the platelet response to acute exercise in healthy adult subjects [106–110]. High levels of plasma fibrinogen after exercise result in elevated blood viscosity and this along with increased vWF binding, αIIbβ3 and P-Selectin expression all contribute to the increased platelet aggregation after acute exercise [111]. In general, it appears that acute vigorous exercise induces a hyper-reactive haemostatic state [112] and a transient increase in agonist-induced platelet adhesion and aggregation *in vitro* and *ex vivo*. However, there is no definitive consensus regarding the short-term effects of exercise on platelet function.

Cardiorespiratory fitness (CRF) is the ability to perform large muscle, moderate to high intensity exercise for prolonged periods and depends on the respiratory, cardiovascular and skeletal systems. CRF represents the adaptation to long-term exercise. High CRF levels are also linked with reduced CVD risk factors such as hypertension, obesity in the general population and CVD patients [113–116]. CRF was first postulated as a significant determinant for changes in platelet function in response to acute exercise after observations that acute strenuous exercise increased platelet activation in sedentary, but not physically active, subjects [106, 117]. The actual relationship between CRF and platelet function has been referred to in a recent breakthrough study by Heber et al., who investigated platelet function and CRF in 62 young women [118]. Platelet function was assessed by determination of P-Selectin and CD40L expression and quantification of platelet ROS generation in platelet-rich plasma (PRP). Basal platelet activation (reflected by CD62P expression) and agonist-induced platelet activation (ROS, CD62P and CD40L) were higher in the LF compared to the MF and HF. The group found no difference between basal CD40L expressions (non-agonist induced). Interestingly, basal platelet function in the MF and HF were almost equal, indicating a definite influence of CRF on platelet function. A high CRF level is a result of exercise training and habitual physical activity. Therefore, research on the effects of longitudinal exercise training on platelet function has mainly shown that habitual exercise has favourable effects on platelet function. Eight weeks of exercise training (60% VO_2 max 5×/week 30 min/day), reduced shear stress-induced platelet activation and ox-LDL-potentiated platelet function [109, 111]. Importantly, after 12 weeks of de-conditioning, the beneficial effects of exercise on platelets were non-existent and platelet function returned to its pre-training state.

De Meirelles et al. reported that chronic physical activity had favourable effects on platelet activation in hypertensive patients at rest [119]. Twelve weeks of regular exercise (75–85% VO_2 max 5×/week for 45–60 min) reduced platelet aggregation in response to collagen. Santilli et al. investigated the effects of regular high intensity (60–75%) aerobic exercise for 2 months in low and intermediate CVD risk sedentary subjects [120]. Exercise training was associated with reductions in TxA_2, plasma P-Selectin and platelet-derived CD40L, despite no reduction in CRP (representing systemic inflammation). Evidently, physical activity and exercise affects nearly all facets of platelet function [121]. Studies on the effects of acute exercise appear to

heighten platelet reactivity. Regular exercise can improve this response, seems to have an antithrombotic effect on platelets and could represent a portion of the protective effects of exercise on CVD risk factors. Moreover, effects of exercise are not maintained with cessation of training. Of importance, all of these studies discussed were performed in adults and not adolescents when the CVD risk factors and atherosclerotic process has begun. Platelet function and exercise in children or adolescents is in its infancy, an area that requires urgent research [122].

2.5. Role of physical inactivity and sedentary lifestyle on platelet function

In contrast to physical activity, physical inactivity/sedentary behaviour is a universal leading cause of death and independent CVD risk factor [123, 124]. Sedentary behaviour refers to any waking activity characterised by an energy expenditure ≤1.5 metabolic equivalents in a sitting or reclining posture [125]. However, in contrast to the evidence supporting the benefits of acute and chronic exercise, relatively little is understood about the mechanisms underlying the physiological, cellular and molecular responses to physical inactivity. Incomplete understanding of this relationship is a huge barrier to combating the development of CVD and its ancillary risk factors. Our knowledge of physical inactivity is somewhat indirect and is mainly based on the positive effects of exercise training on the sedentary population. As a sedentary lifestyle is often associated with obesity [126], some mechanisms involved in the pathogenesis of physical inactivity are similar to that of obesity such as insulin resistance [127], hypertension and increased inflammation [128]. However, distinct factors associated with sedentary behaviour include reduced muscular activity of lower extremities, decreased blood flow and reduction of shear stress, which increases oxidative stress, endothelial dysfunction [129] and arterial remodelling [130, 131].

2.5.1. Physical activity/inactivity-specific miRNA

The plasticity of platelets and other blood cells is vital for responding to environmental changes in response to physical (in)activity patterns. However, the molecular factors influencing platelet function/response/adaptation to physical (in)activity remain poorly understood. Recently identified miRNAs have gained attention as modulators of platelet function [34]. Evidence for miRNA involvement in exercise-associated gene expression changes in a number of cell types including peripheral blood mononuclear cell, neutrophil and skeletal muscle in non-trained and trained subjects has been illustrated [132–134]. Work by Baggish et al. showed altered expression of circulating miRNA (c-miRNA) in response to acute and chronic exercise in athletes [135]. Eight c-miRNA involved in cellular processes related to exercise adaptation (muscle contractility, inflammation, and angiogenesis) were examined. They observed four distinctive signatures of c-miRNA; c-miRNA up-regulated by acute exhaustive exercise pre- and post-exercise intervention, c-miRNA responsive to acute exercise pre- but not post-intervention, c-miRNA only responsive to exercise intervention and non-responsive miRNA. Moreover, evidence of these physical activity-specific microRNA signatures [136–138] has ingrained concepts of physical inactivity-specific miRNA profiles. Epigenetic variation could therefore be a potential mechanism allowing for independent or synergistic effects of physical inactivity on platelet function. Hibler et al. recently described indications for epigenetic variation (by miRNA expression) as a link between physical activity and sedentary lifestyle [139]. An epigenetic adaptation to habitual exercise has been described [140, 141]. Similarly, an epigenetic adaptation to physical inactivity may exist.

2.6. Physical activity/inactivity and platelet epigenetic drift

It has been well recognised that regular exercise may reduce risk of major vascular thrombotic events and protect against CVD [123]. Differences in known factors explain a large percentage of the inverse relationship between physical activity and CVD risk [101, 102]. Nevertheless, over 40% of the inverse association remains unexplained. Although the beneficial effects of regular exercise on blood lipids and blood pressure have been well accepted, research focusing on platelet function has only recently gained greater attention. Whilst it is known that platelet function and platelet indices (markers of platelet activation) are altered in pathological states such as CVD, only a minority of studies have solely examined the relationship between overall physiological health and platelet function in healthy subjects [120, 142]. Therefore, it is imperative that future studies explore the feasibility of platelet indices and whole blood platelet function measurements, as useful, non-invasive initial biomarkers of early/subclinical CVD risk and lifestyle parameters.

Low cardiorespiratory fitness is associated with physical inactivity [143]. This has major health effects globally, with approximately 3.2 million deaths each year attributable to inadequate physical activity. Evidence has shown that physical inactivity and sedentary behaviour have direct effects on CVD risk factors [144]. Moreover, in contrast to the accumulating evidence supporting the benefits of regular exercise, relatively little is understood about the deleterious mechanisms underlying the physiological, cellular and molecular responses to PI, specifically with regard to platelet function.

3. Future research avenues

Our group, in collaboration with the European Space Agency (ESA), the Centre National d'Etudes Spatiales (*CNES*) and *MEDES* (Institute for Space Medicine and Physiology, Toulouse, France) have employed ground-based models of microgravity, i.e., dry water immersion (DI) to study the effects of spaceflight on human physiology in a precisely controlled environment. DI involves immersing a subject in a bath of thermoneutral water covered by a waterproof fabric [145]. Several factors act simultaneously on the human body during immersion, including hydrostatic compression, supportlessness and extensive physical inactivity. Hypokinesia and hypodynamia are the major characteristics of physical inactivity induced by dry immersion. Hypodynamia involves a reduction in postural muscle load, whereas hypokinesia is a decline in motor activity. For these reasons, DI has been well accepted as a valuable tool to study physical inactivity [146]. DI presents a unique opportunity to analyse the specific effects of physical inactivity on platelet physiology/function and related biomarkers.

Recent studies reflect the first comprehensive attempts to evaluate the relationship between platelet function and physical activity, physical inactivity and overweight. While exploratory in nature to date, several questions remain unanswered and so further studies are warranted. The search for simple biomarkers that allow for early identification of subclinical/CVD risk is ongoing. Platelets can reflect changes in unhealthy lifestyle patterns. The Impact-R test is a relatively inexpensive test that can reliably detect changes in platelet adhesion and could be employed for CVD risk evaluation amongst subjects who are asymptomatic. Platelet indices and function markers should be further tested in larger populations to determine their reliability as surrogate markers for evaluating physiological health and to test during either pharmacological and lifestyle interventions. A relatively low dose of exercise has been shown to be

sufficient to normalise platelet function in low fit females [118]. Larger studies incorporating exercise interventions at low doses over a lengthy period of time and examining more extensive aspects of platelet function in low fit subjects would develop this knowledge. The prescription of anti-platelet therapy is frequently used to treat CVD patients. However, the other residual risks (oxidative stress, inflammation etc.), which occur due to associations between CVD risk factors, are not eliminated efficiently by these therapies. In this sense, physical activity has been emphasised as it promotes favourable physiological adaptations, which may attenuate the cardiovascular risk factors and residual risks. Regular exercise may also impact platelet function in CVD patients. Exercise interventions in these populations could be beneficial in terms of reducing anti-platelet therapy dosage or combining anti-platelet therapy with exercise [147], i.e., prescriptive exercise medicine as an adjuvant management strategy/therapy.

The investigation of epigenetic processes is almost a completely unexplored area in platelet biology as analyses of these mechanisms require DNA [2]. Platelets have functionally active mitochondria [148]. Like nuclear DNA, mitochondrial DNA (mtDNA) can also be methylated, moderating control of mitochondrial gene expression. Understanding epigenetic regulation of mitochondrial genes in platelets is proving crucial to understanding their implication in CVD development [6]. Furthermore, miRNA have recently been linked with platelet mitochondrial health in stored platelets [149]. Platelets contain the machinery to process pre-miRNA to mature miRNA [34]. Platelets contain higher levels of pre-miRNA than other blood cells [56], and the maturation of pre-miRNA could contributed to altered miRNA profiles due to physical activity and inactivity. This may represent a more focused and efficient method of monitoring platelet function. Targeting levels of other biogenesis molecules in the miRNA pathway would also be an interesting avenue of platelet miRNA biology. Recently, Elgheznawy et al. showed that Dicer was decreased in patients with TD2M compared to healthy controls, whereas interestingly, Argonaute 2 levels did not differ [150]. Experiments investigating levels of miRNA processing machinery such as Dicer and Argonaute 2 in physically active and sedentary populations would be of major interest.

Long-term lifestyle choices such as physical inactivity may incur epigenetic penalties in megakaryocytes, and in the biological processes of *megakaryocytopoiesis* and *thrombopoiesis*. Thus, platelet miRNA could reflect these epigenetic changes, holding substantial predictive potential of both health and disease. Epigenetic changes in the megakaryocyte genome such as methylation of genes determining platelet biogenesis or changes in histone acetylation with aging have been suggested to play an important role in platelet function [20]. Prescribed exercise could induce epigenetic changes in megakaryocytes to produce a healthier phenotype of platelets with a direct change in platelet reactivity.

4. Conclusion

It is evident that lifestyle factors such as physical activity, physical inactivity and overweight do impact platelet function. Platelets are indeed reflective of physiological and lifestyle changes, making them sensitive biomarkers of human health. Platelets represent a tangible link to physiological and pathological changes within the body. Future research in this area, will no doubt contribute to a greater mechanistic understanding of the relationship between epigenetics, cardiovascular health, lifestyle factors and platelet biology.

Acknowledgements

This work was supported through the Irish Higher Education Authority Programme for Research in Third Level Institutes (HEA-PRTLI Cycle 4: T3 Targeted Therapeutics & Theranostics), the Health Research Board of Ireland (HRB-RP/2005/184, HRA/2009/122, HRA/2009/122/R), Science Foundation Ireland (SFI-04/BR/B0577, 11/TIDA/B1927) to Dr. R.P. Murphy, and the International Visitor Award, Dublin City University, Dublin, Ireland, to Drs. B. Degryse and R.P. Murphy, ASU-DCU Transatlantic Research Fund to M.M and R.P.M. R.P. Murphy is a management committee member of the EU H2020 COST action-CM1406, EpiChemBio. The ground-based protocol and studies of microgravity (3-Day Dry Immersion and 5-Day Dry Immersion) were undertaken in MEDES, the Institute for Space Medicine and Physiology, *Toulouse* University Hospital, France, and was supported and funded by the Centre National d'Etudes Spatiales (*CNES*-National Centre for Space Studies) to M-A.C., R.P.M., N.N., and L.T.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome.

Author details

Laura Twomey[1], Robert G. Wallace[1], Marco Mangone[2], Bernard Degryse[1], Sinead Sheridan[3], Michael Harrison[4], Niall Moyna[1], Gerardene Meade-Murphy[5], Nastassia Navasiolava[6], Marc-Antoine Custaud[6] and Ronan P. Murphy[1]*

*Address all correspondence to: ronan.murphy@dcu.ie

1 School of Health & Human Performance, Dublin City University, Glasnevin, Dublin, Ireland

2 School of Life Sciences, Arizona State University, Tempe Campus, USA

3 School of Public Health, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong

4 School of Health Sciences—Health Sport and Exercise Science, Waterford Institute of Technology, Ireland

5 Department of Pharmacology and Therapeutics, University College Cork, Cork, Ireland

6 Centre de Recherche Clinique du CHU d'Angers, Angers, France

References

[1] Fraga MF, Ballestar E, Paz MF, Ropero S, Setien F, Ballestar ML, et al. Epigenetic differences arise during the lifetime of monozygotic twins. Proceedings of the National Academy of Sciences of the United States of America. 2005;**102**(30):10604-10609

[2] Freson K, Izzi B, Van Geet C. From genetics to epigenetics in platelet research. Thrombosis Research. 2012;**129**(3):325-329

[3] Weinhold B. Epigenetics: The science of change. Environmental Health Perspectives. 2006;**114**(3):A160-A167

[4] Lund G, Zaina S. Atherosclerosis: An epigenetic balancing act that goes wrong. Current Atherosclerosis Reports. 2011;**13**(3):208-214

[5] Zhong Z, Liang S, Sanchez-Lopez E, He F, Shalapour S, Lin XJ, et al. New mitochondrial DNA synthesis enables NLRP3 inflammasome activation. Nature. Aug 2018;**560**(7717): 198-203

[6] Baccarelli AA, Byun HM. Platelet mitochondrial DNA methylation: A potential new marker of cardiovascular disease. Clinical Epigenetics. 2015;**7**:44

[7] Webster AL, Yan MS, Marsden PA. Epigenetics and cardiovascular disease. The Canadian Journal of Cardiology. 2013;**29**(1):46-57

[8] Zaratiegui M, Irvine DV, Martienssen RA. Noncoding RNAs and gene silencing. Cell. 2007;**128**(4):763-776

[9] Calin GA, Liu CG, Ferracin M, Hyslop T, Spizzo R, Sevignani C, et al. Ultraconserved regions encoding ncRNAs are altered in human leukemias and carcinomas. Cancer Cell. 2007;**12**(3):215-229

[10] Sato F, Tsuchiya S, Meltzer SJ, Shimizu K. MicroRNAs and epigenetics. The FEBS Journal. 2011;**278**(10):1598-1609

[11] Gabbianelli R, Damiani E. Epigenetics and neurodegeneration: Role of early-life nutrition. The Journal of Nutritional Biochemistry. 2018;**57**:1-13

[12] Bollati V, Schwartz J, Wright R, Litonjua A, Tarantini L, Suh H, et al. Decline in genomic DNA methylation through aging in a cohort of elderly subjects. Mechanisms of Ageing and Development. 2009;**130**(4):234-239

[13] Tan H, Liu T, Zhang J, Zhou T. Random positioning of nucleosomes enhances heritable bistability. Molecular BioSystems. 2016;**13**(1):132-141

[14] Hannum G, Guinney J, Zhao L, Zhang L, Hughes G, Sadda S, et al. Genome-wide methylation profiles reveal quantitative views of human aging rates. Molecular Cell. 2013;**49**(2): 359-367

[15] Teschendorff AE. Epigenetic aging: Insights from network biology. Aging. 2013;**5**(10): 719-720

[16] Horvath S, Oshima J, Martin GM, Lu AT, Quach A, Cohen H, et al. Epigenetic clock for skin and blood cells applied to Hutchinson Gilford Progeria Syndrome and ex vivo studies. Aging. 2018;**10**(7):1758-1775

[17] Horvath S. DNA methylation age of human tissues and cell types. Genome Biology. 2013;**14**(10):R115

[18] Oshima M, Iwama A. Epigenetics of hematopoietic stem cell aging and disease. International Journal of Hematology. 2014;**100**(4):326-334

[19] Choudry FA, Frontini M. Epigenetic control of haematopoietic stem cell aging and its clinical implications. Stem Cells International. 2016;**2016**:5797521

[20] Daly ME. Determinants of platelet count in humans. Haematologica. 2011;**96**(1):10-13

[21] van Rooij E, Olson EN. MicroRNAs: Powerful new regulators of heart disease and provocative therapeutic targets. The Journal of Clinical Investigation. 2007;**117**(9):2369-2376

[22] van Rooij E, Olson EN. microRNAs put their signatures on the heart. Physiological Genomics. 2007;**31**(3):365-366

[23] Ple H, Landry P, Benham A, Coarfa C, Gunaratne PH, Provost P. The repertoire and features of human platelet microRNAs. PLoS One. 2012;**7**(12):e50746

[24] Laffont B, Corduan A, Ple H, Duchez AC, Cloutier N, Boilard E, et al. Activated platelets can deliver mRNA regulatory Ago2*microRNA complexes to endothelial cells via microparticles. Blood. 2013;**122**(2):253-261

[25] Liang R, Bates DJ, Wang E. Epigenetic control of MicroRNA expression and aging. Current Genomics. 2009;**10**(3):184-193

[26] Bartel DP, Chen CZ. Micromanagers of gene expression: The potentially widespread influence of metazoan microRNAs. Nature Reviews. Genetics. 2004;**5**(5):396-400

[27] Rodriguez A, Griffiths-Jones S, Ashurst JL, Bradley A. Identification of mammalian microRNA host genes and transcription units. Genome Research. 2004;**14**(10A):1902-1910

[28] Bartel DP. MicroRNAs: Target recognition and regulatory functions. Cell. 2009;**136**(2): 215-233

[29] van Rooij E, Sutherland LB, Qi X, Richardson JA, Hill J, Olson EN. Control of stress-dependent cardiac growth and gene expression by a microRNA. Science. 2007;**316**(5824): 575-579

[30] Finnegan EF, Pasquinelli AE. MicroRNA biogenesis: Regulating the regulators. Critical Reviews in Biochemistry and Molecular Biology. 2013;**48**(1):51-68

[31] Gulyaeva LF, Kushlinskiy NE. Regulatory mechanisms of microRNA expression. Journal of Translational Medicine. 2016;**14**(1):143

[32] Bijak M, Saluk J, Ponczek MB, Nowak P, Wachowicz B. The synthesis of proteins in unnucleated blood platelets. Postępy Higieny i Medycyny Doświadczalnej (Online). 2013;**67**:672-679

[33] Thon JN, Devine DV. Translation of glycoprotein IIIa in stored blood platelets. Transfusion. 2007;**47**(12):2260-2270

[34] Landry P, Plante I, Ouellet DL, Perron MP, Rousseau G, Provost P. Existence of a microRNA pathway in anucleate platelets. Nature Structural & Molecular Biology. 2009;**16**(9):961-966

[35] Dangwal S, Thum T. MicroRNAs in platelet physiology and pathology. Hämostaseologie. 2013;**33**(1):17-20

[36] Bray PF, McKenzie SE, Edelstein LC, Nagalla S, Delgrosso K, Ertel A, et al. The complex transcriptional landscape of the anucleate human platelet. BMC Genomics. 2013;**14**:1

[37] Kondkar AA, Bray MS, Leal SM, Nagalla S, Liu DJ, Jin Y, et al. VAMP8/endobrevin is overexpressed in hyperreactive human platelets: Suggested role for platelet microRNA. Journal of Thrombosis and Haemostasis. 2010;**8**(2):369-378

[38] Nagalla S, Shaw C, Kong X, Kondkar AA, Edelstein LC, Ma L, et al. Platelet microRNA-mRNA coexpression profiles correlate with platelet reactivity. Blood. 2011;**117**(19): 5189-5197

[39] Edelstein LC, McKenzie SE, Shaw C, Holinstat MA, Kunapuli SP, Bray PF. MicroRNAs in platelet production and activation. Journal of Thrombosis and Haemostasis. 2013; **11**(Suppl 1):340-350

[40] Simon LM, Edelstein LC, Nagalla S, Woodley AB, Chen ES, Kong X, et al. Human platelet microRNA-mRNA networks associated with age and gender revealed by integrated plateletomics. Blood. 2014;**123**(16):e37-e45

[41] Osman A, Falker K. Characterization of human platelet microRNA by quantitative PCR coupled with an annotation network for predicted target genes. Platelets. 2011;**22**(6): 433-441

[42] Teruel-Montoya R, Kong X, Abraham S, Ma L, Kunapuli SP, Holinstat M, et al. MicroRNA expression differences in human hematopoietic cell lineages enable regulated transgene expression. PLoS One. 2014;**9**(7):e102259

[43] Edelstein LC, Bray PF. MicroRNAs in platelet production and activation. Blood. 2011;**117**(20): 5289-5296

[44] Halkein J, De Windt LJ. miR-223: Sailing to terra incognita for microRNAs in platelets. Thrombosis and Haemostasis. 2013;**110**(6):1112-1113

[45] Shi R, Zhou X, Ji WJ, Zhang YY, Ma YQ, Zhang JQ, et al. The emerging role of miR-223 in platelet reactivity: Implications in antiplatelet therapy. BioMed Research International. 2015;**2015**:981841

[46] Fish JE, Santoro MM, Morton SU, Yu S, Yeh RF, Wythe JD, et al. miR-126 regulates angiogenic signaling and vascular integrity. Developmental Cell. 2008;**15**(2):272-284

[47] Kaudewitz D, Skroblin P, Bender LH, Barwari T, Willeit P, Pechlaner R, et al. Association of MicroRNAs and YRNAs with platelet function. Circulation Research. 2016;**118**(3): 420-432

[48] Emmrich S, Henke K, Hegermann J, Ochs M, Reinhardt D, Klusmann JH. miRNAs can increase the efficiency of ex vivo platelet generation. Annals of Hematology. 2012;**91**(11): 1673-1684

[49] Kamat V, Paluru P, Myint M, French DL, Gadue P, Diamond SL. MicroRNA screen of human embryonic stem cell differentiation reveals miR-105 as an enhancer of megakaryopoiesis from adult CD34+ cells. Stem Cells (Dayton, Ohio). 2014;**32**(5):1337-1346

[50] Zhai PF, Wang F, Su R, Lin HS, Jiang CL, Yang GH, et al. The regulatory roles of microRNA-146b-5p and its target platelet-derived growth factor receptor alpha (PDGFRA) in erythropoiesis and megakaryocytopoiesis. The Journal of Biological Chemistry. 2014;**289**(33): 22600-22613

[51] Chapnik E, Rivkin N, Mildner A, Beck G, Pasvolsky R, Metzl-Raz E, et al. miR-142 orchestrates a network of actin cytoskeleton regulators during megakaryopoiesis. eLife. 2014;**3**:e01964

[52] Hussein K, Theophile K, Buhr T, Beller A, Kreipe H, Bock O. Different lineage involvement in myelodysplastic/myeloproliferative disease with combined MPLW515L and JAK2V617F mutation. British Journal of Haematology. 2009;**145**(5):673-675

[53] Edelstein LC, Simon LM, Montoya RT, Holinstat M, Chen ES, Bergeron A, et al. Racial differences in human platelet PAR4 reactivity reflect expression of PCTP and miR-376c. Nature Medicine. 2013;**19**(12):1609-1616

[54] Diehl P, Fricke A, Sander L, Stamm J, Bassler N, Htun N, et al. Microparticles: Major transport vehicles for distinct microRNAs in circulation. Cardiovascular Research. 2012; **93**(4):633-644

[55] Gidlof O, van der Brug M, Ohman J, Gilje P, Olde B, Wahlestedt C, et al. Platelets activated during myocardial infarction release functional miRNA, which can be taken up by endothelial cells and regulate ICAM1 expression. Blood. 2013;**121**(19):3908-3917, S1-26

[56] Pan Y, Liang H, Liu H, Li D, Chen X, Li L, et al. Platelet-secreted microRNA-223 promotes endothelial cell apoptosis induced by advanced glycation end products via targeting the insulin-like growth factor 1 receptor. Journal of Immunology (Baltimore, Md. : 1950). 2014;**192**(1):437-446

[57] Liang H, Yan X, Pan Y, Wang Y, Wang N, Li L, et al. MicroRNA-223 delivered by platelet-derived microvesicles promotes lung cancer cell invasion via targeting tumor suppressor EPB41L3. Molecular Cancer. 2015;**14**:58

[58] Laffont B, Corduan A, Rousseau M, Duchez AC, Lee CH, Boilard E, et al. Platelet microparticles reprogram macrophage gene expression and function. Thrombosis and Haemostasis. 2016;**115**(2):311-323

[59] O'Donnell CJ, Nabel EG. Cardiovascular genomics, personalized medicine, and the National Heart, Lung, and Blood Institute: Part I: The beginning of an era. Circulation. Cardiovascular Genetics. 2008;**1**(1):51-57

[60] O'Donnell CJ, Nabel EG. Genomics of cardiovascular disease. The New England Journal of Medicine. 2011;**365**(22):2098-2109

[61] Juhola J, Magnussen CG, Viikari JS, Kahonen M, Hutri-Kahonen N, Jula A, et al. Tracking of serum lipid levels, blood pressure, and body mass index from childhood to adulthood:The Cardiovascular Risk in Young Finns Study. The Journal of Pediatrics. 2011; **159**(4):584-590

[62] McNeal CJ, Dajani T, Wilson D, Cassidy-Bushrow AE, Dickerson JB, Ory M. Hypercholesterolemia in youth: Opportunities and obstacles to prevent premature atherosclerotic cardiovascular disease. Current Atherosclerosis Reports. 2010;**12**(1):20-28

[63] Angiolillo DJ, Jakubowski JA, Ferreiro JL, Tello-Montoliu A, Rollini F, Franchi F, et al. Impaired responsiveness to the platelet P2Y12 receptor antagonist clopidogrel in patients with type 2 diabetes and coronary artery disease. Journal of the American College of Cardiology. 2014;**64**(10):1005-1014

[64] Badimon L, Padro T, Vilahur G. Atherosclerosis, platelets and thrombosis in acute ischaemic heart disease. European Heart Journal Acute Cardiovascular Care. 2012;**1**(1):60-74

[65] Daub K, Seizer P, Stellos K, Kramer BF, Bigalke B, Schaller M, et al. Oxidized LDL-activated platelets induce vascular inflammation. Seminars in Thrombosis and Hemostasis. 2010; **36**(2):146-156

[66] Lievens D, Zernecke A, Seijkens T, Soehnlein O, Beckers L, Munnix IC, et al. Platelet CD40L mediates thrombotic and inflammatory processes in atherosclerosis. Blood. 2010;**116**(20):4317-4327

[67] Andre P, Prasad KS, Denis CV, He M, Papalia JM, Hynes RO, et al. CD40L stabilizes arterial thrombi by a beta3 integrin-dependent mechanism. Nature Medicine. 2002;**8**(3): 247-252

[68] Xu XR, Zhang D, Oswald BE, Carrim N, Wang X, Hou Y, et al. Platelets are versatile cells: New discoveries in hemostasis, thrombosis, immune responses, tumor metastasis and beyond. Critical Reviews in Clinical Laboratory Sciences. 2016;**53**(6):409-430

[69] Papp J, Kenyeres P, Toth K. Clinical importance of antiplatelet drugs in cardiovascular diseases. Clinical Hemorheology and Microcirculation. 2013;**53**(1-2):81-96

[70] Metharom P, Berndt MC, Baker RI, Andrews RK. Current state and novel approaches of antiplatelet therapy. Arteriosclerosis, Thrombosis, and Vascular Biology. 2015;**35**(6): 1327-1338

[71] Ward JA, Esa N, Pidikiti R, Freedman JE, Keaney JF, Tanriverdi K, et al. Circulating cell and plasma microRNA profiles differ between non-ST-segment and ST-segment-elevation myocardial infarction. Family Medicine & Medical Science Research. 2013;**2**(2):108

[72] Sondermeijer BM, Bakker A, Halliani A, de Ronde MW, Marquart AA, Tijsen AJ, et al. Platelets in patients with premature coronary artery disease exhibit upregulation of miRNA340* and miRNA624*. PLoS One. 2011;**6**(10):e25946

[73] Zhang YY, Zhou X, Ji WJ, Shi R, Lu RY, Li JL, et al. Decreased circulating microRNA-223 level predicts high on-treatment platelet reactivity in patients with troponin-negative non-ST elevation acute coronary syndrome. Journal of Thrombosis and Thrombolysis. 2014;**38**(1):65-72

[74] Shi R, Ge L, Zhou X, Ji WJ, Lu RY, Zhang YY, et al. Decreased platelet miR-223 expression is associated with high on-clopidogrel platelet reactivity. Thrombosis Research. 2013;**131**(6):508-513

[75] Chen S, Qi X, Chen H, Li M, Gu J, Liu C, et al. Expression of miRNA-26a in platelets is associated with clopidogrel resistance following coronary stenting. Experimental and Therapeutic Medicine. 2016;**12**(1):518-524

[76] Carino A, De Rosa S, Sorrentino S, Polimeni A, Sabatino J, Caiazzo G, et al. Modulation of circulating microRNAs levels during the switch from clopidogrel to ticagrelor. BioMed Research International. 2016;**2016**:3968206

[77] de Boer HC, van Solingen C, Prins J, Duijs JM, Huisman MV, Rabelink TJ, et al. Aspirin treatment hampers the use of plasma microRNA-126 as a biomarker for the progression of vascular disease. European Heart Journal. 2013;**34**(44):3451-3457

[78] Bordeaux BC, Qayyum R, Yanek LR, Vaidya D, Becker LC, Faraday N, et al. Effect of obesity on platelet reactivity and response to low-dose aspirin. Preventive Cardiology. 2010;**13**(2):56-62

[79] Davi G, Guagnano MT, Ciabattoni G, Basili S, Falco A, Marinopiccoli M, et al. Platelet activation in obese women: Role of inflammation and oxidant stress. Journal of the American Medical Association. 2002;**288**(16):2008-2014

[80] Coban E, Ozdogan M, Yazicioglu G, Akcit F. The mean platelet volume in patients with obesity. International Journal of Clinical Practice. 2005;**59**(8):981-982

[81] Samocha-Bonet D, Justo D, Rogowski O, Saar N, Abu-Abeid S, Shenkerman G, et al. Platelet counts and platelet activation markers in obese subjects. Mediators of Inflammation. 2008;**2008**:834153

[82] Csongradi E, Nagy B Jr, Fulop T, Varga Z, Karanyi Z, Magyar MT, et al. Increased levels of platelet activation markers are positively associated with carotid wall thickness and other atherosclerotic risk factors in obese patients. Thrombosis and Haemostasis. 2011;**106**(4):683-692

[83] Cooper JN, Evans RW, Mori Brooks M, Fried L, Holmes C, Barinas-Mitchell E, et al. Associations between arterial stiffness and platelet activation in normotensive overweight and obese young adults. Clinical and Experimental Hypertension (New York, NY: 1993). 2014;**36**(3):115-122

[84] Anfossi G, Russo I, Massucco P, Mattiello L, Doronzo G, De Salve A, et al. Impaired synthesis and action of antiaggregating cyclic nucleotides in platelets from obese

subjects: Possible role in platelet hyperactivation in obesity. European Journal of Clinical Investigation. 2004;**34**(7):482-489

[85] Trovati M, Anfossi G, Massucco P, Mattiello L, Costamagna C, Piretto V, et al. Insulin stimulates nitric oxide synthesis in human platelets and, through nitric oxide, increases platelet concentrations of both guanosine-3′, 5′-cyclic monophosphate and adenosine-3′, 5′-cyclic monophosphate. Diabetes. 1997;**46**(5):742-749

[86] Anfossi G, Russo I, Trovati M. Platelet resistance to the anti-aggregating agents in the insulin resistant states. Current Diabetes Reviews. 2006;**2**(4):409-430

[87] Otani H. Oxidative stress as pathogenesis of cardiovascular risk associated with metabolic syndrome. Antioxidants & Redox Signaling. 2011;**15**(7):1911-1926

[88] Patrono C, Falco A, Davi G. Isoprostane formation and inhibition in atherothrombosis. Current Opinion in Pharmacology. 2005;**5**(2):198-203

[89] Victor VM, Rocha M, Sola E, Banuls C, Garcia-Malpartida K, Hernandez-Mijares A. Oxidative stress, endothelial dysfunction and atherosclerosis. Current Pharmaceutical Design. 2009;**15**(26):2988-3002

[90] Pignatelli P, Sanguigni V, Lenti L, Ferro D, Finocchi A, Rossi P, et al. gp91phox-dependent expression of platelet CD40 ligand. Circulation. 2004;**110**(10):1326-1329

[91] Begonja AJ, Gambaryan S, Geiger J, Aktas B, Pozgajova M, Nieswandt B, et al. Platelet NAD(P)H-oxidase-generated ROS production regulates alphaIIbbeta3-integrin activation independent of the NO/cGMP pathway. Blood. 2005;**106**(8):2757-2760

[92] Leite NR, Siqueira de Medeiros M, Mury WV, Matsuura C, Perszel MB, Noronha Filho G, et al. Platelet hyperaggregability in obesity: Is there a role for nitric oxide impairment and oxidative stress? Clinical and Experimental Pharmacology & Physiology. 2016;**43**(8):738-744

[93] Basili S, Pacini G, Guagnano MT, Manigrasso MR, Santilli F, Pettinella C, et al. Insulin resistance as a determinant of platelet activation in obese women. Journal of the American College of Cardiology. 2006;**48**(12):2531-2538

[94] Russo I, Traversa M, Bonomo K, De Salve A, Mattiello L, Del Mese P, et al. In central obesity, weight loss restores platelet sensitivity to nitric oxide and prostacyclin. Obesity (Silver Spring, Md). 2010;**18**(4):788-797

[95] Vazzana N, Guagnano MT, Cuccurullo C, Ferrante E, Lattanzio S, Liani R, et al. Endogenous secretory RAGE in obese women: Association with platelet activation and oxidative stress. The Journal of Clinical Endocrinology and Metabolism. 2012;**97**(9): E1726-E1730

[96] Keating FK, Schneider DJ, Savage PD, Bunn JY, Harvey-Berino J, Ludlow M, et al. Effect of exercise training and weight loss on platelet reactivity in overweight patients with coronary artery disease. Journal of Cardiopulmonary Rehabilitation and Prevention. 2013;**33**(6):371-377

[97] Blokhin IO, Lentz SR. Mechanisms of thrombosis in obesity. Current Opinion in Hematology. 2013;**20**(5):437-444

[98] Freedman JE, Larson MG, Tanriverdi K, O'Donnell CJ, Morin K, Hakanson AS, et al. Relation of platelet and leukocyte inflammatory transcripts to body mass index in the Framingham heart study. Circulation. 2010;**122**(2):119-129

[99] Zampetaki A, Kiechl S, Drozdov I, Willeit P, Mayr U, Prokopi M, et al. Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. Circulation Research. 2010;**107**(6):810-817

[100] Li J, Siegrist J. Physical activity and risk of cardiovascular disease—A meta-analysis of prospective cohort studies. International Journal of Environmental Research and Public Health. 2012;**9**(2):391-407

[101] Mora S, Cook N, Buring JE, Ridker PM, Lee IM. Physical activity and reduced risk of cardiovascular events: Potential mediating mechanisms. Circulation. 2007;**116**(19): 2110-2118

[102] Kwasniewska M, Kostka T, Jegier A, Dziankowska-Zaborszczyk E, Leszczynska J, Rebowska E, et al. Regular physical activity and cardiovascular biomarkers in prevention of atherosclerosis in men: A 25-year prospective cohort study. BMC Cardiovascular Disorders. 2016;**16**:65

[103] Kumar R, Bouskill V, Schneiderman JE, Pluthero FG, Kahr WH, Craik A, et al. Impact of aerobic exercise on haemostatic indices in paediatric patients with haemophilia. Thrombosis and Haemostasis. 2016;**115**(6):1120-1128

[104] Shepard RJ. Evaluation of the Canadian home fitness test in middle-aged men. Canadian Medical Association Journal. 1977;**117**(10):1136-1138

[105] Aurigemma C, Fattorossi A, Sestito A, Sgueglia GA, Farnetti S, Buzzonetti A, et al. Relationship between changes in platelet reactivity and changes in platelet receptor expression induced by physical exercise. Thrombosis Research. 2007;**120**(6):901-909

[106] Wang JS, Jen CJ, Kung HC, Lin LJ, Hsiue TR, Chen HI. Different effects of strenuous exercise and moderate exercise on platelet function in men. Circulation. 1994;**90**(6):2877-2885

[107] Wang JS, Jen CJ, Chen HI. Effects of chronic exercise and deconditioning on platelet function in women. Journal of Applied Physiology (Bethesda, Md: 1985). 1997;**83**(6): 2080-2085

[108] Wang JS, Yang CF, Wong MK, Chow SE, Chen JK. Effect of strenuous arm exercise on oxidized-LDL-potentiated platelet activation in individuals with spinal cord injury. Thrombosis and Haemostasis. 2000;**84**(1):118-123

[109] Wang JS, Li YS, Chen JC, Chen YW. Effects of exercise training and deconditioning on platelet aggregation induced by alternating shear stress in men. Arteriosclerosis, Thrombosis, and Vascular Biology. 2005;**25**(2):454-460

[110] Wang JS. Exercise prescription and thrombogenesis. Journal of Biomedical Science. 2006;**13**(6):753-761

[111] Wang JS, Chow SE. Effects of exercise training and detraining on oxidized low-density lipoprotein-potentiated platelet function in men. Archives of Physical Medicine and Rehabilitation. 2004;**85**(9):1531-1537

[112] Huskens D, Roest M, Remijn JA, Konings J, Kremers RM, Bloemen S, et al. Strenuous exercise induces a hyperreactive rebalanced haemostatic state that is more pronounced in men. Thrombosis and Haemostasis. 2016;**115**(6):1109-1119

[113] Franklin BA, Lavie CJ, Squires RW, Milani RV. Exercise-based cardiac rehabilitation and improvements in cardiorespiratory fitness: Implications regarding patient benefit. Mayo Clinic Proceedings. 2013;**88**(5):431-437

[114] Swift DL, Lavie CJ, Johannsen NM, Arena R, Earnest CP, O'Keefe JH, et al. Physical activity, cardiorespiratory fitness, and exercise training in primary and secondary coronary prevention. Circulation Journal: Official Journal of the Japanese Circulation Society. 2013;**77**(2):281-292

[115] DeFina LF, Haskell WL, Willis BL, Barlow CE, Finley CE, Levine BD, et al. Physical activity versus cardiorespiratory fitness: Two (partly) distinct components of cardiovascular health? Progress in Cardiovascular Diseases. 2015;**57**(4):324-329

[116] Myers J, McAuley P, Lavie CJ, Despres JP, Arena R, Kokkinos P. Physical activity and cardiorespiratory fitness as major markers of cardiovascular risk: Their independent and interwoven importance to health status. Progress in Cardiovascular Diseases. 2015;**57**(4):306-314

[117] Kestin AS, Ellis PA, Barnard MR, Errichetti A, Rosner BA, Michelson AD. Effect of strenuous exercise on platelet activation state and reactivity. Circulation. 1993;**88**(4 Pt 1): 1502-1511

[118] Heber S, Assinger A, Pokan R, Volf I. Correlation between cardiorespiratory fitness and platelet function in healthy women. Medicine and Science in Sports and Exercise. 2016;**48**(6):1101-1110

[119] de Meirelles LR, Mendes-Ribeiro AC, Mendes MA, da Silva MN, Ellory JC, Mann GE, et al. Chronic exercise reduces platelet activation in hypertension: upregulation of the L-arginine-nitric oxide pathway. Scandinavian Journal of Medicine & Science in Sports. 2009;**19**(1):67-74

[120] Santilli F, Vazzana N, Liani R, Guagnano MT, Davi G. Platelet activation in obesity and metabolic syndrome. Obesity Reviews: An Official Journal of the International Association for the Study of Obesity. 2012;**13**(1):27-42

[121] Heber S, Volf I. Effects of physical (in)activity on platelet function. BioMed Research International. 2015;**2015**:165078

[122] Ribeiro J, Almeida-Dias A, Ascensao A, Magalhaes J, Oliveira AR, Carlson J, et al. Hemostatic response to acute physical exercise in healthy adolescents. Journal of Science and Medicine in Sport. 2007;**10**(3):164-169

[123] Blair SN, Morris JN. Healthy hearts—And the universal benefits of being physically active: Physical activity and health. Annals of Epidemiology. 2009;**19**(4):253-256

[124] Kohl HW 3rd, Craig CL, Lambert EV, Inoue S, Alkandari JR, Leetongin G, et al. The pandemic of physical inactivity: Global action for public health. Lancet (London, England). 2012;**380**(9838):294-305

[125] Chevance G, Foucaut AM, Bernard P. State of knowledge on sedentary behaviors. Presse Medicale (Paris, France: 1983). 2016;**45**(3):313-318

[126] Van Gaal LF, Mertens IL, De Block CE. Mechanisms linking obesity with cardiovascular disease. Nature. 2006;**444**(7121):875-880

[127] Hamburg NM, McMackin CJ, Huang AL, Shenouda SM, Widlansky ME, Schulz E, et al. Physical inactivity rapidly induces insulin resistance and microvascular dysfunction in healthy volunteers. Arteriosclerosis, Thrombosis, and Vascular Biology. 2007;**27**(12): 2650-2656

[128] Nosova EV, Yen P, Chong KC, Alley HF, Stock EO, Quinn A, et al. Short-term physical inactivity impairs vascular function. The Journal of Surgical Research. 2014;**190**(2): 672-682

[129] Thosar SS, Johnson BD, Johnston JD, Wallace JP. Sitting and endothelial dysfunction: The role of shear stress. Medical Science Monitor: International Medical Journal of Experimental and Clinical Research. 2012;**18**(12):RA173-RA180

[130] Thijssen DH, Maiorana AJ, O'Driscoll G, Cable NT, Hopman MT, Green DJ. Impact of inactivity and exercise on the vasculature in humans. European Journal of Applied Physiology. 2010;**108**(5):845-875

[131] Thijssen DH, Dawson EA, van den Munckhof IC, Birk GK, Timothy Cable N, Green DJ. Local and systemic effects of leg cycling training on arterial wall thickness in healthy humans. Atherosclerosis. 2013;**229**(2):282-286

[132] Radom-Aizik S, Zaldivar F Jr, Oliver S, Galassetti P, Cooper DM. Evidence for microRNA involvement in exercise-associated neutrophil gene expression changes. Journal of Applied Physiology (Bethesda, Md: 1985). 2010;**109**(1):252-261

[133] Radom-Aizik S, Zaldivar F Jr, Leu SY, Adams GR, Oliver S, Cooper DM. Effects of exercise on microRNA expression in young males peripheral blood mononuclear cells. Clinical and Translational Science. 2012;**5**(1):32-38

[134] Nielsen S, Akerstrom T, Rinnov A, Yfanti C, Scheele C, Pedersen BK, et al. The miRNA plasma signature in response to acute aerobic exercise and endurance training. PLoS One. 2014;**9**(2):e87308

[135] Baggish AL, Hale A, Weiner RB, Lewis GD, Systrom D, Wang F, et al. Dynamic regulation of circulating microRNA during acute exhaustive exercise and sustained aerobic exercise training. The Journal of Physiology. 2011;**589**(Pt 16):3983-3994

[136] Bye A, Rosjo H, Aspenes ST, Condorelli G, Omland T, Wisloff U. Circulating microRNAs and aerobic fitness—The HUNT-Study. PLoS One. 2013;**8**(2):e57496

[137] Kangas R, Pollanen E, Rippo MR, Lanzarini C, Prattichizzo F, Niskala P, et al. Circulating miR-21, miR-146a and Fas ligand respond to postmenopausal estrogen-based hormone replacement therapy—A study with monozygotic twin pairs. Mechanisms of Ageing and Development. 2014;**143-144**:1-8

[138] Altana V, Geretto M, Pulliero A. MicroRNAs and physical activity. MicroRNA (Shariqah, United Arab Emirates). 2015;**4**(2):74-85

[139] Hibler E. Epigenetics and colorectal neoplasia: The evidence for physical activity and sedentary behavior. Current Colorectal Cancer Reports. 2015;**11**(6):388-396

[140] Ling C, Ronn T. Epigenetic adaptation to regular exercise in humans. Drug Discovery Today. 2014;**19**(7):1015-1018

[141] Pareja-Galeano H, Sanchis-Gomar F, Garcia-Gimenez JL. Physical exercise and epigenetic modulation: Elucidating intricate mechanisms. Sports Medicine (Auckland, NZ). 2014;**44**(4):429-436

[142] Santilli F, Vazzana N, Iodice P, Lattanzio S, Liani R, Bellomo RG, et al. Effects of high-amount-high-intensity exercise on in vivo platelet activation: Modulation by lipid peroxidation and AGE/RAGE axis. Thrombosis and Haemostasis. 2013;**110**(6):1232-1240

[143] Wei M, Gibbons LW, Kampert JB, Nichaman MZ, Blair SN. Low cardiorespiratory fitness and physical inactivity as predictors of mortality in men with type 2 diabetes. Annals of Internal Medicine. 2000;**132**(8):605-611

[144] Prentice A, Jebb S. TV and inactivity are separate contributors to metabolic risk factors in children. PLoS Medicine. 2006;**3**(12):e481

[145] Coupe M, Fortrat JO, Larina I, Gauquelin-Koch G, Gharib C, Custaud MA. Cardiovascular deconditioning: From autonomic nervous system to microvascular dysfunctions. Respiratory Physiology & Neurobiology. 2009;**169**(Suppl 1):S10-S12

[146] Widlansky ME. The danger of sedenterism: Endothelium at risk. American Journal of Physiology. Heart and Circulatory Physiology. 2010;**299**(2):H243-H244

[147] Martins CC, Bagatini MD, Cardoso AM, Zanini D, Abdalla FH, Baldissarelli J, et al. Regular exercise training reverses ectonucleotidase alterations and reduces hyperaggregation of platelets in metabolic syndrome patients. Clinica Chimica Acta; International Journal of Clinical Chemistry. 2016;**454**:66-71

[148] Antony PM, Boyd O, Trefois C, Ammerlaan W, Ostaszewski M, Baumuratov AS, et al. Platelet mitochondrial membrane potential in Parkinson's disease. Annals of Clinical Translational Neurology. 2015;**2**(1):67-73

[149] Dahiya N, Sarachana T, Kulkarni S, Wood WH III, Zhang Y, Becker KG, et al. miR-570 interacts with mitochondrial ATPase subunit g (ATP5L) encoding mRNA in stored platelets. Platelets. 2017;**28**(1):74-81

[150] Elgheznawy A, Shi L, Hu J, Wittig I, Laban H, Pircher J, et al. Dicer cleavage by calpain determines platelet microRNA levels and function in diabetes. Circulation Research. 2015;**117**(2):157-165

Section 4

Homeostasis and Disease

Chapter 7

Reactive Oxygen Species, Cellular Redox Homeostasis and Cancer

Rabiatul Basria S.M.N. Mydin and Simon I. Okekpa

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76096

Abstract

Redox homeostasis is attained by the cautious regulation of both reactive oxygen species (ROS) formation and removal from the body system. A shift in ROS balance promotes oxidative injury and tumour development by inflicting damage to DNA and inducing inconsistencies in the genome. The sources of endogenous ROS in a cell include mETC, NOX, LOX, cytochrome P450 and XO. The exogenous risk factors of ROS are pollutants, chemicals/drugs, radiation and heavy metals. Oxidative phosphorylation in the mitochondria produces ROS with unpaired electrons. Superoxide anion is the major ROS produced in the human mitochondria. Bulk of the ROS generation in the mitochondria occurs at the electron transport chain as derivatives of respiration. Cancer cells sustain ROS production by suppressing the antioxidant-generation system. Balance between ROS production and subsequent detoxification is regulated by scavenging enzymes and antioxidant agents. Failure in sirtuin-3 (SIRT3), ATM and p53 activities elevates the intracellular levels of ROS. PKCα induces the expression of NOX (DUOX) during cancer development and the consequent increase in ROS production. The PI3K/AKT signalling pathway activates NOX with consequent ROS production and subsequent induction of instability in the genome, leading to cancer. In conclusion, the interruption of the redox pathways that regulate ROS and its redox signalling activities affects cell physiology and can ultimately result in abnormal signalling, uncontrolled oxidative impairment and tumorigenesis.

Keywords: homeostasis, cancer, reactive oxygen species, mitochondrial electron transport chain (ETC), NOX, GSH, glutathione oxidase (GPX), superoxide dismutase (SOD), thioredoxin (TRX), sirtuins

1. Introduction

Reactive oxygen species (ROS) are known as oxygen free radicals, which greatly contribute in complex cellular pathways, such as metabolism, immune system regulation, proliferation, differentiation and vascular transformations [1, 2]. ROS have a short life span and possess unpaired electrons [3]. Oxidative stress, DNA damage and cancer occur as a result of ROS imbalance due to dysregulated generation of free radicals (ROS) from oxygen and inability to neutralise and detoxify the harmful effects caused by the free radicals in the body through counteracting their oxidative effect by antioxidants [4–6]. Under normal and healthy circumstances, ROS production and removal are strictly regulated and controlled by very effective defensive machinery that blocks excessive ROS production. Some ROS, such as superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2), are necessary life functions because they play a vital role in the regulation of cell defence mechanisms necessary for signalling, steroid synthesis, G-protein-coupled-receptor activation, gene expression and transcription factor regulation [7, 8]. Therefore, ROS can act both as good and bad molecules because of their dual nature and can either induce regulation of cellular physiology or promote the induction of cytotoxicity depending on generation levels, site of generation and magnitude of generation [9]. However, high ROS levels make cells vulnerable to damage. The derivatives from oxygen contain free radicals, such as superoxide anion (O_2^-) and hydroxyl radical (OH^-) plus non-radical molecules, such as hypochlorous acid and H_2O_2 [3], which have been linked to oxidative injury due to their high reactivity potential against proteins, lipids and DNA [10].

The generation of ROS can be activated by either various endogenous or exogenous factors. The major source of endogenous ROS in cells of mammals is the mitochondrial electron transport chain (ETC). Other endogenous ROS sources are from the activities of NADPH oxidases (NOX), lipoxygenases (LOX), cytochrome P450 and xanthine oxidase (XO) [11]. Exogenous factors that contribute to ROS production are pollutants, chemicals/drugs, radiation and heavy metals [12]. Redox homeostasis is attained by cautious regulation of both ROS formation and removal from the body system [10]. Maintenance of homeostasis and signalling event of redox require the significant regulation of synthesis and detoxification. An interruption to the redox route that regulates ROS and its redox signalling activities affects cell physiology and can ultimately result in abnormal signalling, uncontrolled toxic by-product accumulation, oxidative impairment and cytotoxicity [2]. High oxidative stress levels are normally linked to abnormalities, which characterise tumour-specific modification that exposes the cancer cells to additional raise of ROS depending on the strength of their antioxidant defence system [13].

Cancer is one of the leading causes of death globally. Recent evidence suggests altered redox stability and dysregulated redox signalling as the two frequent hallmarks of cancers, which are implicated in the progression of malignancy and treatment resistance [13]. Cancer cells have been postulated to persistently exhibit high levels of reactive oxygen species (ROS) as a result of alterations in microenvironment, genetic mutations and dysregulation of metabolic processes [13]. This shift in pro-oxidant balance promotes tumour development by inflicting damage to DNA and causing inconsistency in the genome [1]. The DNA damage and instability induced to the genome activate an inflammatory reaction leading to stability of hypoxia

inducible factor-1 and subsequent metabolic reprogramming [13, 14]. The ROS detoxification mechanism has provided selective advantage for its survival during pro-oxidation situations. Balance between ROS production and its quick detoxification are regulated by scavenging enzymes and antioxidant agents that limit the accumulation of ROS in the body.

2. Roles of mitochondria in ROS formation

Mitochondria generates 90% of the energy required for cells and tissues to function effectively and serves as the core site for energy metabolism in the cells, since it is involved in the generation of ATP via oxidative phosphorylation (OXPHOS) [15]. This process liberates electron from reducing substrates and delivers the electron to O_2 leading to the establishment of electrochemical gradient which triggers the ATP synthesis. Oxidative phosphorylation in the mitochondria produces ROS with unpaired electrons due to electron reduction from the oxygen [16–18]. Superoxide anion ($O_2^{-}\bullet$) is the major ROS produced in the human mitochondria which is formed due to monoelectronic O_2 reduction. Most ROS originate from the superoxide anion which also mediates oxidative chain reactions.

In vivo production of $O_2^{-}\bullet$ could either be synthesised enzymatically by cyP450-dependent oxygenases, NADPH oxidase and xanthine oxidase or non-enzymatically by transferring an electron directly to O_2 [18]. $O_2^{-}\bullet$ is capable of reacting with free radicals such as nitric oxide ($NO\bullet$) to produce reactive nitrogen species (RNS) [19]. $O_2^{-}\bullet$ dismutation can occur spontaneously or through superoxide dismutases (SODs) catalysed reaction to generate hydrogen peroxide (H_2O_2) [20–22]. The mitochondrial generated H_2O_2 has numerous probable fates. H_2O_2 is fairly stable and permeable to the membrane, and therefore, it can diffuse inside the cell and get eliminated by mitochondrial or cytosolic antioxidant systems, which are catalase, thioredoxin-peroxidase and glutathione-peroxidase [23]. Mitochondrially produced H_2O_2, also function as a cytosolic signalling molecule, thereby, affecting the networks that control energy metabolism, stress response, redox balance and cell cycle [24–26]. None metabolised H_2O_2 in the mitochondria undergoes Fenton reaction and then transformed subsequently into hydroxyl radical ($\bullet OH$) which is naturally a very strong oxidant with high damaging impact on molecules due to its high reactive nature [27]. The above reason has made researchers to believe that mitochondria have developed competent systems for H_2O_2 removal and also mechanisms for metal chelating (chaperone proteins) which prevents the formation of radical. Bulk of the ROS generation in the mitochondria occurs at the electron transport chain (ETC) as derivatives of respiration [17, 18, 28]. The ETC terminal component known as cytochrome c oxidase (Complex IV) acquires four (4) electrons from cytochrome c and then reduces one molecule of O_2 to form two H_2O. All the intermediates that is partially reduced are retained until reduction is fully achieved [16].

2.1. ROS and mitochondrial activation of apoptosis

High exposure of the mitochondria to ROS results to injurious consequences such as inflicting oxidative mitochondrial DNA damage. It has also been suggested that ROS is deeply involved

in the extrinsic pathway of apoptosis. Extrinsic receptor-mediated pathway for cell death requires active engagement of the death receptors on the cell membrane surface with their corresponding ligands [29]. Receptor-mediated apoptotic pathway comprises of death receptors such as CD95 (Fas), TNF-related apoptosis-inducing ligand (TRAIL) receptors and TNF. Activation of Fas as well as TNFR1 generate ROS due to superoxide ($O_2\bullet-$) production and formation of NADPH oxidase daises derived from lipid raft. Induction of apoptosis or necrosis is linked with lipid raft-mediated downstream ROS generation [30, 31]. Downregulation of FLIP (FLICE inhibitory protein), a strong inhibitor of apoptosis is mediated by ROS via ubiquitination and consequent proteasome degradation or by scavenging of nitric oxide (NO) to prevent FLIP S-nitrosation and cytoprotection [32]. ROS sensitises cancer cells to apoptosis induced by TRAIL [33]. CD95 and TRAIL death receptors have been observed to be highly upregulated in reaction in the presence of hydrogen peroxide via NF-kappa B activation [34]. ROS promote apoptosis via JNK activation, inducing either intrinsic or extrinsic apoptotic signalling [35]. TNFα induced ROS perpetrates oxidation of JNK, thereby, inactivating-phosphatases via catalytic transformation of their cysteine into sulfenic acid resulting to prolonged activation of JNK which is necessary for the release of cytochrome c and cleavage of caspase 3 as well as cell death [36]. TNFα activates MAPK cascade. ASK1, a redox-sensitive MAPK kinase, is located at the JNK upstream. Reduced thioredoxin1 (Trx1) binds to ASK1 during non-oxidising circumstances to form a complex known as ASK1 signalosome (Trx1/ASK1 complex) which perform redox switch functions. Persistent cellular ROS causes detachment of the oxidised Trx1 from the Trx1/ASK1 complex leading to full ASK1 activation through TRAF2/6 recruitment [37]. ASK2 a member of ASK family attaches to ASK1 and stabilises it in mitochondria, nucleus and cytosol. Saxena et al. have revealed that redox protein known as thioredoxin interacting protein (TXNIP) with apoptosis promoting potential under oxidative stress, shuttles from the nucleus to the mitochondria leading to the removal of TXNIP from ASK1 and formation of a compound with mitochondrial Trx2. This suppression of the inhibition is mediated by Trx2 results in ASK1phosphorylation and induction of the mitochondrial pathway for apoptosis with caspase-3 cleavage and cytochrome c release [38]. The major target for ROS inside the mitochondria is the permeability transition pore (mPTP) in which the oxidative modification of its proteins has significant influence on the anion fluxes within the mitochondria [39]. This could cause overload of Ca^{2+} and ROS in reaction to pro-apoptotic stimuli causing mPTP to assume a very high state of conductance allowing unrestrained entry of solutes along the electrochemical gradient into the matrix of the mitochondria. The above phenomenon is termed mitochondrial permeability transition (MPT), which results in mitochondrial membrane potential dissipation and consequent osmotic swelling of the matrix of the mitochondria due to fluid influx [40]. The early phase of the mitochondrial swelling involves water movement from inter-cristae spaces into the mitochondrial matrix. Persistent movement of this water exacts pressure on the outer membrane due to increased volume of the matrix leading to mPTP opening and/or rupturing of the outer membrane of the mitochondria allowing the matrix to expand further [41]. This causes cytochrome c to be released with consequent activation of the downstream effector caspases by Apaf-1-pro-caspase 9-apoptosome complex.

http://dx.doi.org/10.5772/intechopen.76096

3. Antioxidant system responsible for the redox homeostasis

The antioxidant systems are either enzymatic or non-enzymatic. The enzymatic antioxidant system consists of peroxiredoxin (Prx) system, catalase, SOD and the glutathione peroxidase (GPx) system, while the non-enzymatic antioxidant systems consist of α-tocopherol, lipoic acid and ascorbic acid [42–45].

3.1. Superoxide dismutases (SOD)

Intracellular ROS levels are regulated by the balance between ROS generating enzymes and antioxidant enzymes, which include superoxide dismutases (SOD), catalase, thioredoxin and glutathione peroxidase (GPX) [42]. SOD functions to convert O_2^- into H_2O_2, which is later converted into water by glutathione peroxidase or catalase. Human cells express three types of SOD: MnSOD (manganese SOD) expressed by the mitochondria, CuZnSOD (copper-zinc SOD) expressed by the cytoplasm and third is the extracellular SOD. A study has demonstrated that lack of MnSOD in mice generated excessive oxidative stress causing their mortality [46]. Another study also revealed that mice with a deficiency of CuZnSOD developed hepatocellular carcinoma due to sustained oxidative damage [47]. Lack of MnSOD has also been linked to elevated risk of lung cancer, prostate cancer, non-Hodgkin's lymphoma and ovarian cancer [48–51].

3.2. Glutathione oxidase (GPX)

GPX is a selenium-dependent antioxidant enzyme, which regulates hydrogen and lipid peroxide levels. Lack of GPX in the body increases tissue damage by ROS [43] and low GPX levels, which results in increased LDL oxidation [44]. GPX catalyses the reduction of hydrogen peroxide to form glutathione disulphide (GSSG) with glutathione (GSH) functioning as the substrate. An increased risk of bladder cancer, lung cancer and breast cancer has been associated with the substitution of proline-leucine at codon 198 in human GPX [52–55].

3.3. Thioredoxins (Trx)

The protective function of thioredoxins (Trxs) in cells against oxidative stress is via the reaction between their active site known as 2-cysteine and ROS resulting in reduction of oxidised proteins. Trxs also function as hydrogen donors to thioredoxin-dependent peroxide reductases. Trx possesses a Cys-Gly-Pro-Cys active site, which is essential for redox regulatory functions of Trx. Trx, when combined with Trx reductase and NADPH, forms a redox-sensitive machinery, which controls the levels of oxidised cysteine on proteins. The antioxidant properties of Trx can be attributed to the reduction of the oxidised form of Trx peroxidase by Trx, while the reduced peroxidase scavenges H_2O_2 [45]. The two isoforms of Trx are Trx1 (expressed in the cytoplasm and the nucleus) and Trx2 (expressed in the mitochondria), which are very crucial for cell survival [56]. Trx1 is a redox-sensitive binding protein that controls the

activity of NF-κB through the reduction of cys62 on the p50 of NF-κB [57]. Trx1 has been linked to breast tumours, colon cancer, cervical cancer, gastric cancer, lung cancer, liver cancer and melanoma and carcinomas of the pancreas [58–62].

3.4. GSH

GSH is a thiol protein consisting of cysteine, glutamine and glycine, which functions as an important antioxidant in detoxification of metabolic processes [63]. Elevated levels of GSH in the cancerous patient tissue are reinforced by improved accessibility to the biosynthetic

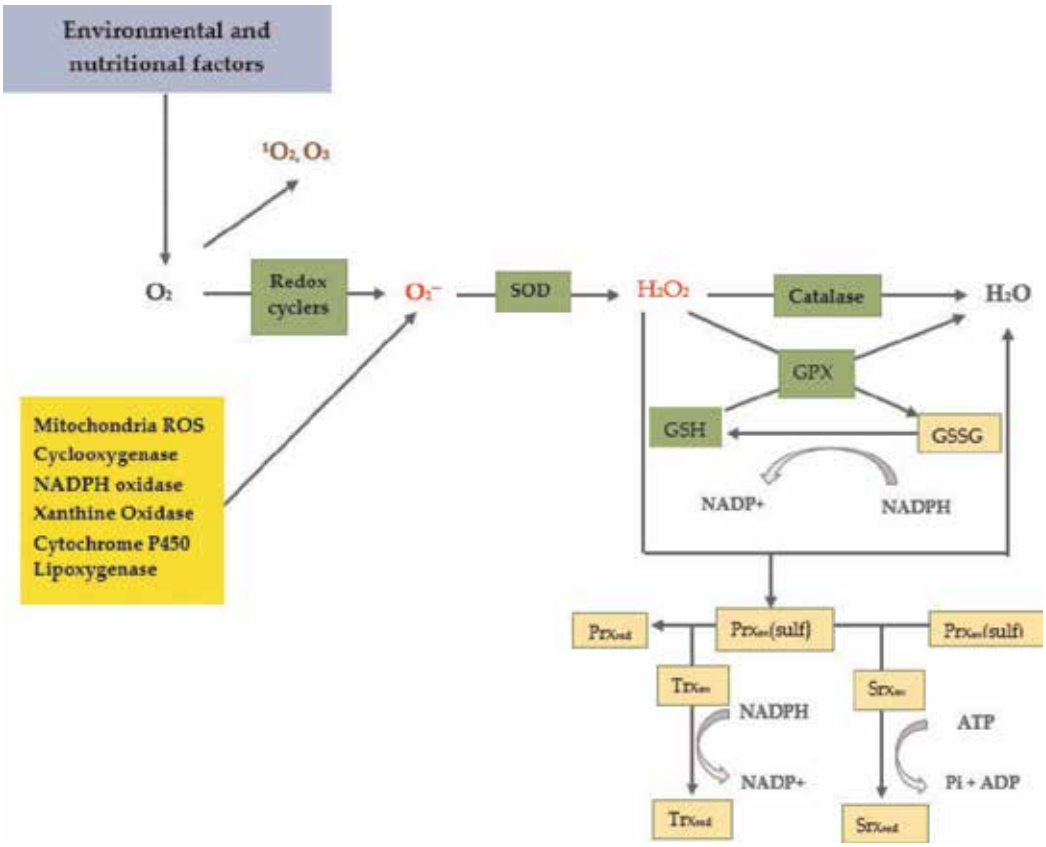

Figure 1. Mechanism of ROS formation and disposal. Enzymatically or non-enzymatically generated superoxide reacts with other radicals. The generated superoxide and H_2O_2 form hydroxyl radicals and singlet oxygen. SODs catalyse the dismutation of the superoxide, while H_2O_2 decomposition to water is catalysed by catalases in conjunction with glutathione peroxidase/GPx and Prxs. Thiol, a component of cysteine found in Prx, is oxidatively converted into Cys-sulfenic acid by (Prx_{ox}) and subsequently reduced by thioredoxin (Trx_{red}). H_2O_2 further oxidises Cys-sulfenic acid (Prx_{ox}) into Cys-sulfinic acid and reduced subsequently back by ATP and reduced sulfiredoxin (Srx_{red}) to Cys-sulfenic acid (Cys-SOH). Cys-SOH formed functions to regulate protein activities by absorbing the oxidative insults leading to the deflection of injurious oxidative impairment [24]. Cys-SOH can be converted into oxidative post-translational modification (Ox-PTM) in redox environment. This modification reacts with tripeptide glutathione (Glu-Cys-Gly) to form S-glutathionylated Cys (GSSG) or react with thiol to form disulphide bond. Formation of GSSG protects the Cys of the host from further oxidative reactions.

elements of GSH, such as glutamate, cysteine and glycine [64, 65]. The negative regulator of cysteine/glutamate known as SLC7A11 is always upregulated in human tumours [66]. Glutamate cysteine ligase modifier subunit (GCLM) is also upregulated in many types of human cancer but also requisite for effective GSH synthesis [67]. The cellular levels of GSH and its regeneration are modulated by NADPH and GR catalysing the reduction of oxidised GSSG back to GSH, in a process facilitated by the upregulation of NADPH production by cancer cells (**Figure 1**). Maintenance and elevation of GSH levels in cells are critical for the initiation and proliferation of tumours [67, 68]. Loss of GSH, or decrease in the ratio of glutathione to glutathione disulphide (GSH:GSSG), results in increased oxidative stress susceptibility and cancer development. Also, elevated levels of GSH increase antioxidant activities against numerous cancer cells, thereby enhancing the resistance of the cancer cells against oxidative stress [69].

3.5. Peroxiredoxins (Prxs)

Prxs are made up of six isoenzyme families capable of reducing H_2O_2 and alkyl hydroperoxides to their resultant H_2O or alcohol. Prxs are essential antioxidants that mediate the balancing mechanism of cellular H_2O_2 production, which is necessary for signalling and cell metabolism [70]. Nrf2 upregulates Prxs in oxidative stress circumstances [71]. PRDX1 plays the role of tumour suppressor in the development of breast cancer by interacting with oncogene (c-Myc) suppressing its transcriptional action [72, 73]. Contrarily, PRDX1 has promotional activities on pancreatic carcinoma, hepatocellular cancer, oesophageal cancer, oral cancer and lung cancer via upregulation of heme-oxygenase 1 and NF-κB pathway activation [74–77]. PRDX2 stimulates colorectal carcinoma by upregulating Wnt/β catenin levels, while it stimulates prostate cancer by upregulating the receptive activities of androgen [78, 79].

4. Enzymes responsible for the redox homeostasis

Some of the enzymes associated with redox homeostasis are NADPH oxidase, ATM kinase and sirtuin-3 among others.

4.1. NADPH oxidase

NADPH oxidase is hetero-proteins, which consist of seven isoforms. ROS production by NADPH oxidase is via the NOX protein. NADPH oxidases are referred collectively as the NOX family. The NOX family is comprised of NOX (NOX1, NOX2, NOX3, NOX4 andNOX5) and dual oxidases (DUOX1 and DUOX2) [80–82]. The isoforms DUOX1 and DUOX2 contain additional peroxidase domains, which exert the catalytic dismutation of superoxide anion to yield H_2O_2 [74]. Cytosolic electron transfer from NADPH across the cell membrane is catalysed by NADPH oxidase, thereby oxidising the molecular oxygen, which is later reduced to generate ROS species called superoxide anion radical (O_2^-). Generation of NADPH in the mitochondria plays a critical role in metastasis. In many tumour cells, reductive carboxylation in the mitochondria to generate NADPH is powered by mitochondrial citrate transporter

(mCTP), cytosolic isocitrate dehydrogenase (IDH1) and mitochondrial isocitrate dehydrogenase (IDH2). This development assists cells to retain redox balance in the mitochondria averting the oxidative trauma received as a result of the detachment from the extracellular matrix [75].

4.2. ATM kinase

ROS production can be inhibited by ATM kinase and the same ATM kinase also serves as the function of redox-regulated DNA damage sensing protein [76]. ATM-regulated tumour suppressor works by interfering with KEAP1-facilitated NRF2 ubiquitination, thereby activating and stabilising the major regulators of antioxidants [77]. ATM facilitates the upregulation of glucose-6-phosphate dehydrogenase in order to promote NADPH production, thereby suppressing ROS levels [83].

4.3. Sirtuin-3

ROS levels are inherently elevated in cancer cells owing to mitochondrial defective oxidative metabolism [84]. Upregulated oxidative signals are implicated in the development and advancement of different cancer types [85]. Raised levels of ROS contribute to the initiation of cancer, transformation to malignancy and therapy resistance. ROS inflicted damage is more frequently seen in mitochondrial DNA than nuclear DNA because of its closeness to the main ROS source of generation and inadequate restoring capacities. For instance, silent information regulators of gene transcription-3 (sirtuin-3) are crucial in ROS regulation and effective flow of electrons via ETC. Failure in sirtuin-3 activities elevates intracellular levels of ROS, thereby inducing instability to the DNA of the mitochondria [86]. Sirtuins are an enzyme family, which is dependent on NAD-class III histone deacetylase. Seven homologues of Sirtuins (SIRT1–7) exist in mammals [87]. SIRT1 deacetylate gene regulates proteins like p53, forkhead proteins and NF-κB, which modulate resistance of cells to stress [36]. The deacetylase function of sirtuin proteins depends on the intracellular or endogenous content of NAD^+ [87]. Sirtuins are involved in the catalysis of exclusive reactions that lead to the formation of deacetylated substrate, acetyl ADP-ribose (AADPR) and nicotinamide [87]. Also, SIRT1 interrupts apoptosis, rescuing vulnerable cells after repetitive oxidative stress exposure [60]. SIRT2 deacetylate cytoskeletal proteins, such as forkhead proteins, histones, etc. SIRT3 reacts to redox status changes in the mitochondria by influencing the enzyme activities of manganese superoxide dismutase (MnSOD), which in turn scavenges ROS in the mitochondria and thus modulating the levels of ROS and metabolic homeostatic reliability [87].

5. Pathways implicated in redox homeostasis

Dysregulations associated with various tumour proliferations, autophagy and apoptosis depend on the activation of targets sensitive to redox reactions, such as PKC, Akt, PTEN, p53, etc. [88].

5.1. PKC pathways

PKC has isoenzymes, such as PKCα, PKCβ and PKCδ, with conflicting actions in different cancers [89]. PKCβ is the isoenzyme of PKC responsible for the stimulation and phosphorylation of p66/shc, which binds to cytochrome c in order to activate ROS generation [90]. Recent studies demonstrated that PKCα induces the expression of DUOX (a member of NOX family) during cancer development and subsequent ROS production [91, 92]. PKCδ has been demonstrated to be involved in the activation of NOX through the alteration of redox balance, thereby influencing the differentiation of tumour cells [90].

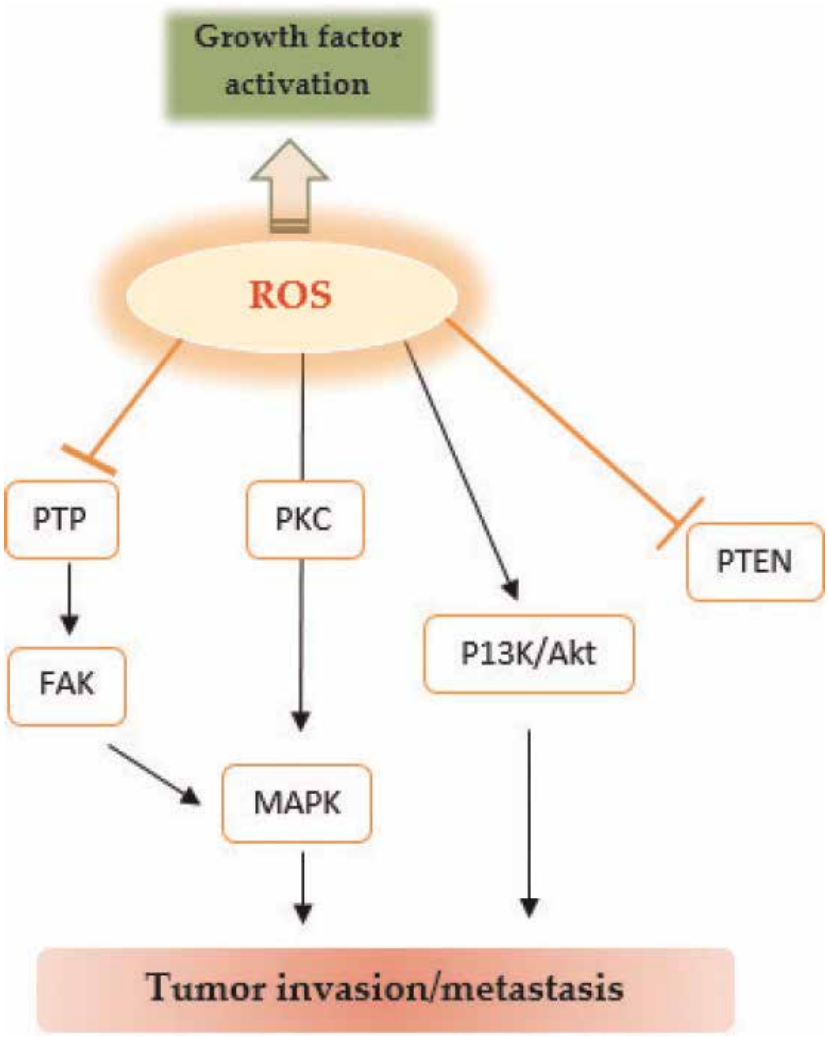

Figure 2. Effect of ROS imbalance in some pathways. Imbalance in the levels of ROS in the cell causes inhibition of PTEN and PTP dependent phosphorylation and consequent inactivation of FAK. The PI3K/Akt and PKC signals are activated in the process leading to invasion/metastasis.

5.2. PI3K/AKT pathway

PI3K/AKT signalling pathway activates NOX with consequent ROS production and subsequent induction of instability to the genome of cancer cells [91]. Upregulation of PTEN suppresses ROS synthesis, thus regulating PI3K/AKT pathway [92]. ROS-mediated PTEN inactivity alters kinase-phosphatase stability favouring the signalling of tumorigenic-tyrosine kinase receptor via Akt (**Figure 2**) leading to the inhibition of apoptosis due to phosphorylation and inactivation of Bad and caspase-9 [93]. Akt improves cell survival by negatively regulating the activities of Bcl-2 homology domain 3 (BH3)-only proteins via binding and inactivation of pro-survival Bcl-2 family members. Akt survival effects on cells depend on the S136 phosphorylation on BAD [94]. Akt-mediated BAD phosphorylation is stimulated by survival factors on S136 leading to the creation of 14-3-3 protein binding site causing BAD to miss its protein target [94]. Akt phosphorylates FOXO proteins (FOXO1, FOXO3a and FOXO4) on T24 and S256 attaching onto 14-3-3 proteins in the cell nucleus causing displacement of transcription factors of FOXO from their gene target and consequent export out of the nucleus. This results in the blocking FOXO facilitated transcription of genes that can stimulate apoptotic processes and cell-cycle arrest, thereby encouraging cell survival. Akt also promotes survival by targeting HDM2 causing inhibition of BH3-only proteins by triggering degradation of p53. Akt induces phosphorylation of HDM2 on S166 and S186, causing HDM2 translocation to the nucleus to regulate p53 function negatively [94]. Deficiency of p53 in cancer results in higher cytokine transcription and consequent accumulation of ROS [95]. p53 is another tumour suppressor that has the potential to activate NRF2 and elevate antioxidant enzyme (SOD, GPX1 and NADPH) expression, thereby reactivating the antioxidant system (**Figure 1**) [10, 96]. Previous study has shown that 53 plays a pro oxidant role through the reduction of SLC7A11 expression, which is responsible for cysteine uptake during GSH synthesis [87]. Thus, the antioxidant activity of p53 is necessary because of its ability to avert cancer, thus implicating loss of tumour suppressors in upregulated intracellular ROS expression.

6. Conclusion

Redox homeostasis is achieved by the regulation of both ROS formation and removal. Shifts in ROS balance induce oxidative injury and tumour development. The balance between ROS production and subsequent detoxification is regulated by scavenging enzymes and antioxidant agents. Targeting the ROS generation pathway with anticancer medications can aid patient recuperation. Therefore, the modulation of ROS levels is a modern anticancer therapy. Further studies are needed to determine when ROS inhibition and activation can be applied in clinical cancer treatment.

Acknowledgements

The authors would like to thank Universiti Sains Malaysia Bridging Grant: 304/CIPPT/6316184 and Dr. Norehan Mokhtar, The Director of Advanced Medical and Dental Institute, Universiti Sains Malaysia for supporting this work.

http://dx.doi.org/10.5772/intechopen.76096

Conflict of interest

All authors declared that there is no conflict of interests.

Abbreviations

ROS	reactive oxygen species
SOD	superoxide dismutases
TRX	thioredoxin
GPX	glutathione oxidase
GSH	glutathione
mETC	mitochondrial electron transport chain
NOX	NADPH oxidases
LOX	lipoxygenases
XO	xanthine oxidase
Prx	peroxiredoxin
MnSOD	manganese SOD
CuZnSOD	copper-zinc SOD
GCLM	glutamate cysteine ligase modifier subunit
GSSG	S-glutathionylated Cys
PRDXs	peroxiredoxins
GR	glutathione reductase
GCLM	glutamate cysteine ligase modifier subunit
Ox-PTM	oxidative post-translational modification
DUOX	dual oxidases
mCTP	mitochondrial citrate transporter
IDH	isocitrate dehydrogenase
Sirtuin-3	silent information regulators of gene transcription-3
ATM	ataxia telangiectasia mutated
FAK	focal adhesion kinase
BAD	Bcl-2-associated death

FOXO	forkhead box
PI3K	phosphoinositide-3 kinase
PTP	protein tyrosine phosphatase
PTEN	phosphatase and tensin homologue
PKC	protein kinase C
Nrf2	nuclear factor erythroid 2-related factor 2

Author details

Rabiatul Basria S.M.N. Mydin[1]* and Simon I. Okekpa[1,2]

*Address all correspondence to: rabiatulbasria@usm.my

1 Oncological and Radiological Sciences Cluster, Advanced Medical and Dental Institute, Universiti Sains Malaysia, Kepala Batas, Pulau Pinang Malaysia

2 Department of Medical Laboratory Science, Faculty of Health Sciences, Ebonyi State University, Abakaliki, Ebonyi State, Nigeria

References

[1] Chouchani ET, Pell VR, Gaude E, Aksentijević D, Sundier SY, Robb EL, et al. Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. Nature. 2014;**515**(7527):431

[2] Holmström KM, Finkel T. Cellular mechanisms and physiological consequences of redox-dependent signalling. Nature Reviews. Molecular Cell Biology. 2014;**15**(6):411

[3] Manda G, Isvoranu G, Comanescu MV, Manea A, Butuner BD, Korkmaz KS. The redox biology network in cancer pathophysiology and therapeutics. Redox Biology. 2015;**5**: 347-357

[4] Schramm A, Matusik P, Osmenda G, Guzik TJ. Targeting NADPH oxidases in vascular pharmacology. Vascular Pharmacology. 2012;**56**(5–6):216-231

[5] Cretu E, Trifan A, Aprotosoaie AC, Miron A. 15-Lipoxygenase inhibition, superoxide and hydroxyl radicals scavenging activities of *Cedrus brevifolia* bark extracts. Revista Medico-Chirurgicală a Societăţii de Medici şi Naturalişti din Iaşi. 2013;**117**:250-256

[6] Ciocoiu M, Miron A, Bădescu M. New polyphenolic extracts for oxidative stress treatment in experimental diabetes. Revista Medico-Chirurgicală a Societăţii de Medici şi Naturalişti din Iaşi. 2008;**112**(3):757-763

[7] Amanso AM, Griendling KK. Differential roles of NADPH oxidases in vascular physiology and pathophysiology. Frontiers in Bioscience (Scholar Edition). 2012;**4**:1044

[8] Sedeek M, Nasrallah R, Touyz RM, Hébert RL. NADPH oxidases, reactive oxygen species, and the kidney: Friend and foe. Journal of the American Society of Nephrology. 2013; **24**(10):1512-1518

[9] Bar-Peled L, Kemper EK, Suciu RM, Vinogradova EV, Backus KM, Horning BD, et al. Chemical proteomics identifies druggable vulnerabilities in a genetically defined cancer. Cell. 2017;**171**(3):696-709

[10] Kong H, Chandel NS. Regulation of redox balance in cancer and T cells. Journal of Biological Chemistry. 2018;**293**(20):7499-7507

[11] Li X, Fang P, Mai J, Choi ET, Wang H, Yang XF. Targeting mitochondrial reactive oxygen species as novel therapy for inflammatory diseases and cancers. Journal of Hematology & Oncology. 2013;**6**(1):19

[12] Galadari S, Rahman A, Pallichankandy S, Thayyullathil F. Reactive oxygen species and cancer paradox: To promote or to suppress? Free Radical Biology and Medicine. 2017;**104**: 144-164

[13] Panieri E, Santoro MM. ROS homeostasis and metabolism: A dangerous liason in cancer cells. Cell Death & Disease. 2016;**7**(6):e2253

[14] Gao P, Tchernyshyov I, Chang TC, Lee YS, Kita K, Ochi T, et al. c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. Nature. 2009;**458**(7239):762

[15] Brown GC. Control of respiration and ATP synthesis in mammalian mitochondria and cells. The Biochemical Journal. 1992;**284**(1):1-3

[16] Kowaltowski AJ, de Souza-Pinto NC, Castilho RF, Vercesi AE. Mitochondria and reactive oxygen species. Free Radical Biology & Medicine. 2009;**47**(4):333-343

[17] Liu Y, Fiskum G, Schubert D. Generation of reactive oxygen species by the mitochondrial electron transport chain. Journal of Neurochemistry. 2002;**80**(5):780-787

[18] Turrens JF. Mitochondrial formation of reactive oxygen species. The Journal of Physiology. 2003;**52**(2):335-344

[19] Radi R, Cassina A, Hodara R. Nitric oxide and peroxynitrite interactions with mitochondria. Biological Chemistry. 2002;**383**(3–4):401-409

[20] Loschen G, Azzi A, Richter C, Flohé L. Superoxide radicals as precursors of mitochondrial hydrogen peroxide. FEBS Letters. 1974;**42**(1):68-72

[21] Weisiger RA, Fridovich I. Superoxide dismutase organelle specificity. The Journal of Biological Chemistry. 1973;**248**(10):3582-3592

[22] Boveris A, Chance B. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. The Biochemical Journal. 1973;**134**(3):707-716

[23] Nordberg J, Arner ES. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system1. Free Radical Biology & Medicine. 2001;**31**(11):1287-1312

[24] Droge W. Free radicals in the physiological control of cell function. Physiological Reviews. 2002;**82**(1):47-95

[25] Hamanaka RB, Chandel NS. Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes. Trends in Biochemical Sciences. 2010;**35**(9):505-513

[26] Starkov AA. The role of mitochondria in reactive oxygen species metabolism and signaling. Annals of the New York Academy of Sciences. 2008;**1147**(1):37-52

[27] Aruoma OI. Free radicals, oxidative stress, and antioxidants in human health and disease. Journal of the American Oil Chemists' Society. 1998;**75**(2):199-212

[28] Murphy MP. How mitochondria produce reactive oxygen species. The Biochemical Journal. 2009;**417**(1):1-3

[29] Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, et al. Two CD95 (APO-1/Fas) signaling pathways. The EMBO Journal. 1998;**17**(6):1675-1687

[30] Morgan MJ, Liu ZG. Reactive oxygen species in TNFα-induced signaling and cell death. Molecules and Cells. 2010;**30**(1):1-2

[31] Li PL, Gulbins E. Lipid rafts and redox signaling. Antioxidants & Redox Signaling. 2007; **9**(9):1411-1416

[32] Wang L, Azad N, Kongkaneramit L, Chen F, Lu Y, Jiang BH, et al. The Fas death signaling pathway connecting reactive oxygen species generation and FLICE inhibitory protein down-regulation. The Journal of Immunology. 2008;**180**(5):3072-3080

[33] Izeradjene K, Douglas L, Tillman DM, Delaney AB, Houghton JA. Reactive oxygen species regulate caspase activation in tumor necrosis factor–related apoptosis-inducing ligand–resistant human colon carcinoma cell lines. Cancer Research. 2005;**65**(16):7436-7445

[34] Woo SH, Park IC, Park MJ, An S, Lee HC, Jin HO, et al. Arsenic trioxide sensitizes CD95/Fas-induced apoptosis through ROS-mediated upregulation of CD95/Fas by NF-κB activation. International Journal of Cancer. 2004;**112**(4):596-606

[35] Pantano C, Shrivastava P, McElhinney B, Janssen-Heininger Y. Hydrogen peroxide signaling through tumor necrosis factor receptor 1 leads to selective activation of c-Jun N-terminal kinase. The Journal of Biological Chemistry. 2003;**278**(45):44091-44096

[36] Kamata H, Honda SI, Maeda S, Chang L, Hirata H, Karin M. Reactive oxygen species promote TNFα-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. Cell. 2005;**120**(5):649-661

[37] Fujino G, Noguchi T, Matsuzawa A, Yamauchi S, Saitoh M, Takeda K, et al. Thioredoxin and TRAF family proteins regulate reactive oxygen species-dependent activation of ASK1 through reciprocal modulation of the N-terminal homophilic interaction of ASK1. Molecular and Cellular Biology. 2007;**27**(23):8152-8163

[38] Saxena G, Chen J, Shalev A. Intracellular shuttling and mitochondrial function of thioredoxin-interacting protein. Journal of Biological Chemistry. 2010;**285**(6):3997-4005

[39] Zoratti M, Szabò I. The mitochondrial permeability transition. Biochimica et Biophysica Acta (BBA)–Reviews on Biomembranes. 1995;**1241**(2):139-176

[40] Fulda S, Galluzzi L, Kroemer G. Targeting mitochondria for cancer therapy. Nature Reviews. Drug Discovery. 2010;**9**(6):447

[41] Kaasik A, Safiulina D, Zharkovsky A, Veksler V. Regulation of mitochondrial matrix volume. American Journal of Physiology. Cell Physiology. 2007;**292**(1):C157-C163

[42] Kim HW, Lin A, Guldberg RE, Ushio-Fukai M, Fukai T. Essential role of extracellular SOD in reparative neovascularization induced by hindlimb ischemia. Circulation Research. 2007;**101**(4):409-419

[43] Lewis P, Stefanovic N, Pete J, Calkin AC, Giunti S, Thallas-Bonke V, et al. Lack of the antioxidant enzyme glutathione peroxidase-1 accelerates atherosclerosis in diabetic apolipoprotein e–deficient mice. Circulation. 2007;**115**(16):2178-2187

[44] Hamanishi T, Furuta H, Kato H, Doi A, Tamai M, Shimomura H, et al. Functional variants in the glutathione peroxidase-1 (GPx-1) gene are associated with increased intima-media thickness of carotid arteries and risk of macrovascular diseases in Japanese type 2 diabetic patients. Diabetes. 2004;**53**(9):2455-2460

[45] Kang SW, Chae HZ, Seo MS, Kim K, Baines IC, Rhee SG. Mammalian peroxiredoxin isoforms can reduce hydrogen peroxide generated in response to growth factors and tumor necrosis factor-α. The Journal of Biological Chemistry. 1998;**273**(11):6297-6302

[46] Li Y, Huang TT, Carlson EJ, Melov S, Ursell PC, Olson JL, et al. Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. Nature Genetics. 1995;**11**(4):376

[47] Pham CG, Bubici C, Zazzeroni F, Papa S, Jones J, Alvarez K, et al. Ferritin heavy chain upregulation by NF-κB inhibits TNFα-induced apoptosis by suppressing reactive oxygen species. Cell. 2004;**119**(4):529-542

[48] Liu G, Zhou W, Wang LI, Park S, Miller DP, Xu LL, et al. MPO and SOD2 polymorphisms, gender, and the risk of non-small cell lung carcinoma. Cancer Letters. 2004;**214**(1):69-79

[49] Kang D, Lee KM, Park SK, Berndt SI, Peters U, Reding D, et al. Functional variant of manganese superoxide dismutase (SOD2 V16A) polymorphism is associated with prostate cancer risk in the prostate, lung, colorectal, and ovarian cancer study. Cancer Epidemiology and Prevention Biomarkers. 2007;**16**(8):1581-1586

[50] Wang SS, Davis S, Cerhan JR, Hartge P, Severson RK, Cozen W, et al. Polymorphisms in oxidative stress genes and risk for non-Hodgkin lymphoma. Carcinogenesis. 2006;**27**(9): 1828-1834

[51] Olson SH, Carlson MD, Ostrer H, Harlap S, Stone A, Winters M, et al. Genetic variants in SOD2, MPO, and NQO1, and risk of ovarian cancer. Gynecologic Oncology. 2004;**93**(3): 615-620

[52] Ichimura Y, Habuchi T, Tsuchiya N, Wang L, Oyama C, Sato K, et al. Increased risk of bladder cancer associated with a glutathione peroxidase 1 codon 198 variant. The Journal of Urology. 2004;**172**(2):728-732

[53] Raaschou-Nielsen O, Sørensen M, Hansen RD, Frederiksen K, Tjønneland A, Overvad K, et al. GPX1 Pro198Leu polymorphism, interactions with smoking and alcohol consumption, and risk for lung cancer. Cancer Letters. 2007;**247**(2):293-300

[54] Ravn-Haren G, Olsen A, Tjønneland A, Dragsted LO, Nexø BA, Wallin H, et al. Associations between GPX1 Pro198Leu polymorphism, erythrocyte GPX activity, alcohol consumption and breast cancer risk in a prospective cohort study. Carcinogenesis. 2005;**27**(4):820-825

[55] Arsova-Sarafinovska Z, Matevska N, Eken A, Petrovski D, Banev S, Dzikova S. Glutathione peroxidase 1 (GPX1) genetic polymorphism, erythrocyte GPX activity, and prostate cancer risk. International Urology and Nephrology. 2009;**41**(1):63

[56] Tanaka T, Hosoi F, Yamaguchi-Iwai Y, Nakamura H, Masutani H, Ueda S, et al. Thioredoxin-2 (TRX-2) is an essential gene regulating mitochondria-dependent apoptosis. The EMBO Journal. 2002;**21**(7):1695-1703

[57] Matthews JR, Wakasugi N, Virelizier JL, Yodoi J, Hay RT. Thiordoxin regulates the DNA binding activity of NF-χB by reduction of a disulphid bond involving cysteine 62. Nucleic Acids Research. 1992;**20**(15):3821-3830

[58] Turunen N, Karihtala P, MÄntyniemi A, Sormunen R, Holmgren A, Kinnula VL, et al. Thioredoxin is associated with proliferation, p53 expression and negative estrogen and progesterone receptor status in breast carcinoma. APMIS. 2004;**112**(2):123-132

[59] Fujii S, Nanbu Y, Nonogaki H, Konishi I, Mori T, Masutani H, et al. Coexpression of adult T-cell leukemia-derived factor, a human thioredoxin homologue, and human papillomavirus DNA in neoplastic cervical squamous epithelium. Cancer. 1991;**68**(7):1583-1591

[60] Nakamura H, Masutani H, Tagaya Y, Yamauchi A, Inamoto T, Nanbu Y, et al. Expression and growth-promoting effect of adult t-cell leukemia-derived factor a human thioredoxin homologue in hepatocellular carcinoma. Cancer. 1992;**69**(8):2091-2097

[61] Soini Y, Kahlos K, Näpänkangas U, Kaarteenaho-Wiik R, Säily M, Koistinen P, et al. Widespread expression of thioredoxin and thioredoxin reductase in non-small cell lung carcinoma. Clinical Cancer Research. 2001;**7**(6):1750-1757

[62] Lincoln DT, Ali EE, Tonissen KF, Clarke FM. The thioredoxin-thioredoxin reductase system: Over-expression in human cancer. Anticancer Research. 2003;**23**(3B):2425-2433

[63] Sies H. Glutathione and its role in cellular functions. Free Radical Biology & Medicine. 1999;**7**(9–10):916-921

[64] Quinlan CL, Orr AL, Perevoshchikova IV, Treberg JR, Ackrell BA, Brand MD. Mitochondrial complex II can generate reactive oxygen species at high rates in both the forward and reverse reactions. The Journal of Biological Chemistry. 2012;**287**(32):27255-27264

[65] Gamcsik MP, Kasibhatla MS, Teeter SD, Colvin OM. Glutathione levels in human tumors. Biomarkers. 2012;**17**(8):671-691

[66] Jiang L, Kon N, Li T, Wang SJ, Su T, Hibshoosh H, et al. Ferroptosis as a p53-mediated activity during tumour suppression. Nature. 2015;**520**(7545):57

[67] Harris IS, Treloar AE, Inoue S, Sasaki M, Gorrini C, Lee KC, et al. Glutathione and thioredoxin antioxidant pathways synergize to drive cancer initiation and progression. Cancer Cell. 2015;**27**(2):211-222

[68] Cramer SL, Saha A, Liu J, Tadi S, Tiziani S, Yan W, et al. Systemic depletion of L-cyst (e) ine with cyst (e) inase increases reactive oxygen species and suppresses tumor growth. Nature Medicine. 2017;**23**(1):120

[69] Traverso N, Ricciarelli R, Nitti M, Marengo B, Furfaro AL, Pronzato MA, et al. Role of glutathione in cancer progression and chemoresistance. Oxidative Medicine and Cellular Longevity. 2013;**2013**:972913

[70] Perkins A, Nelson KJ, Parsonage D, Poole LB, Karplus PA. Peroxiredoxins: Guardians against oxidative stress and modulators of peroxide signaling. Trends in Biochemical Sciences. 2015;**40**(8):435-445

[71] Park MH, Jo M, Kim YR, Lee CK, Hong JT. Roles of peroxiredoxins in cancer, neurodegenerative diseases and inflammatory diseases. Pharmacology & Therapeutics. 2016;**163**:1-23

[72] Egler RA, Fernandes E, Rothermund K, Sereika S, de Souza-Pinto N, Jaruga P, et al. Regulation of reactive oxygen species, DNA damage, and c-Myc function by peroxiredoxin 1. Oncogene. 2005;**24**(54):8038

[73] Cao J, Schulte J, Knight A, Leslie NR, Zagozdzon A, Bronson R, et al. Prdx1 inhibits tumorigenesis via regulating PTEN/AKT activity. The EMBO Journal. 2009;**28**(10):1505-1517

[74] Sumimoto H. Structure, regulation and evolution of Nox-family NADPH oxidases that produce reactive oxygen species. The FEBS Journal. 2008;**275**(13):3249-3277

[75] Segal AW. The function of the NADPH oxidase of phagocytes and its relationship to other NOXs in plants, invertebrates, and mammals. The International Journal of Biochemistry & Cell Biology. 2008;**40**(4):604-618

[76] Jiang L, Shestov AA, Swain P, Yang C, Parker SJ, Wang QA, et al. Reductive carboxylation supports redox homeostasis during anchorage-independent growth. Nature. 2016; **532**(7598):255

[77] D D'Souza A, Parish IA, Krause DS, Kaech SM, Shadel GS. Reducing mitochondrial ROS improves disease-related pathology in a mouse model of ataxia-telangiectasia. Molecular Therapy. 2013;**21**(1):42-48

[78] Lu W, Fu Z, Wang H, Feng J, Wei J, Guo J. Peroxiredoxin 2 is upregulated in colorectal cancer and contributes to colorectal cancer cells' survival by protecting cells from oxidative stress. Molecular and Cellular Biochemistry. 2014;**387**(1–2):261-270

[79] Shiota M, Yokomizo A, Kashiwagi E, Takeuchi A, Fujimoto N, Uchiumi T, et al. Peroxiredoxin 2 in the nucleus and cytoplasm distinctly regulates androgen receptor activity in prostate cancer cells. Free Radical Biology & Medicine. 2011;**51**(1):78-87

[80] Cramer SL, Saha A, Liu J, Tadi S, Tiziani S, Yan W, et al. Systemic depletion of serum l-Cyst (e) ine with an engineered human enzyme induces production of reactive oxygen species and suppresses tumor growth in mice. Nature Medicine. 2017;**23**(1):120

[81] Segal BH, Veys P, Malech H, Cowan MJ. Chronic granulomatous disease: Lessons from a rare disorder. Biology of Blood and Marrow Transplantation. 2011;**17**(1):S123-S131

[82] Lassègue B, San Martín A, Griendling KK. Biochemistry, physiology, and pathophysiology of NADPH oxidases in the cardiovascular system. Circulation Research. 2012;**110**(10):1364-1390

[83] Gorrini C, Baniasadi PS, Harris IS, Silvester J, Inoue S, Snow B, et al. BRCA1 interacts with Nrf2 to regulate antioxidant signaling and cell survival. The Journal of Experimental Medicine. 2013;**210**(8):1529-1544

[84] Cosentino C, Grieco D, Costanzo V. ATM activates the pentose phosphate pathway promoting anti-oxidant defence and DNA repair. The EMBO Journal. 2011;**30**(3):546-555

[85] Tafani M, Sansone L, Limana F, Arcangeli T, De Santis E, Polese M, et al. The interplay of reactive oxygen species, hypoxia, inflammation, and sirtuins in cancer initiation and progression. Oxidative Medicine and Cellular Longevity. 2016;**2016**:3907147

[86] Prasad S, Gupta SC, Tyagi AK. Reactive oxygen species (ROS) and cancer: Role of antioxidative nutraceuticals. Cancer Letters. 2017;**387**:95-105

[87] Haigis MC, Deng CX, Finley LW, Kim HS, Gius D. SIRT3 is a mitochondrial tumor suppressor: A scientific tale that connects aberrant cellular ROS, the Warburg effect, and carcinogenesis. Cancer Research. 2012;**72**(10):2468-2472

[88] Braidy N, Poljak A, Grant R, Jayasena T, Mansour H, Chan-Ling T, et al. Differential expression of sirtuins in the aging rat brain. Frontiers in Cellular Neuroscience. 2015;**9**:167

[89] Marengo B, Nitti M, Furfaro AL, Colla R, Ciucis CD, Marinari UM, Pronzato MA, Traverso N, Domenicotti C. Redox homeostasis and cellular antioxidant systems: Crucial players in cancer growth and therapy. Oxidative Medicine and Cellular Longevity. 2016; **2016**:6235641

[90] Antal CE, Hudson AM, Kang E, Zanca C, Wirth C, Stephenson NL, et al. Cancer-associated protein kinase C mutations reveal kinase's role as tumor suppressor. Cell. 2015;**160**(3):489-502

[91] Wang J, Shao M, Liu M, Peng P, Li L, Wu W, et al. PKCα promotes generation of reactive oxygen species via DUOX2 in hepatocellular carcinoma. Biochemical and Biophysical Research Communications. 2015;**463**(4):839-845

[92] Nitti M, Furfaro AL, Cevasco C, Traverso N, Marinari UM, Pronzato MA, et al. PKC delta and NADPH oxidase in retinoic acid-induced neuroblastoma cell differentiation. Cellular Signalling. 2010;**22**(5):828-835

[93] Leonarduzzi G, Sottero B, Gabriella Testa G, Biasi F, Poli G. New insights into redox-modulated cell signaling. Current Pharmaceutical Design. 2011;**17**(36):3994-4006

[94] Xu J, Tian W, Ma X, Guo J, Shi Q, Jin Y, et al. The molecular mechanism underlying morphine-induced Akt activation: Roles of protein phosphatases and reactive oxygen species. Cell Biochemistry and Biophysics. 2011;**61**(2):303-311

[95] Manning BD, Cantley LC. AKT/PKB signaling: Navigating downstream. Cell. 2007;**129**(7): 1261-1274

[96] Chen W, Sun Z, Wang XJ, Jiang T, Huang Z, Fang D, et al. Direct interaction between Nrf2 and p21 Cip1/WAF1 upregulates the Nrf2-mediated antioxidant response. Molecular Cell. 2009;**34**(6):663-673

Chapter 8

S6 Kinase: A Compelling Prospect for Therapeutic Interventions

Sheikh Tahir Majeed, Rabiya Majeed,
Ghazia Shah and Khurshid I Andrabi

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.75209

Abstract

S6 kinase, a member of AGC family of protein kinases and a downstream effector of mTORC1 pathway has over the years found much relevance in maintaining a normal cellular state by virtue of its established role in regulation of cell growth and proliferation. S6 kinase activity has been linked to different cellular processes like glucose homeostasis, translational and transcriptional regulation. Hence any dysregulation in S6K1 leads to the emergence of various pathological conditions like diabetes, cancer and obesity. It is as such S6 kinase has emerged as a potential target for therapeutic interventions employed in curing such diseases. The Present Chapter reviews the regulation of S6K1, its structural organization and functions, besides highlighting its potential to act as an alternative therapeutic target for various cancerous situations exhibiting deranged mTOR signaling so as to overcome the possibility of relapses observed otherwise while using conventional drugs

Keywords: S6 kinase, mTOR, rapamycin, cancer, cell growth and proliferation

1. Introduction

Cell signaling pathways function as cohorts to contribute in maintaining cellular homeostasis-a phenomenon that remains pivotal in regulating cell growth and survival. The unique but immensely coordinated response of these diverse signals is primarily regulated by the availability of nutrients, growth factors and energy status of the cell. The signals destined to activate certain cellular functions may simultaneously be antagonistic for other functions; to strike a much required balance for proper functioning of the cell. Relentless efforts

over the last several decades have led to recognize the ability of these signaling networks to promote cell growth and proliferation by way of their potential to phosphorylate one another to distinguish themselves as kinases. The kinases specifically transfer the phosphoryl group from ATP to their substrates such as to bring about a change in their function that helps transduce the upstream signals for the accomplishment of functions. Intrigued by the properties of these kinases, the scientific community over the years has shown great interest in identifying more kinases that lead to the identification of a family of protein kinases known as ribosomal S6 kinases (RSKs) that respond to nutrients and growth factors. The RSKs came to fore after studying the inducible phosphorylations on ribosomal protein S6 (rps6) and accordingly p90 ribosomal S6 kinase was identified from Xenopus oocytes. The homologous counterparts of p90 S6 kinase in humans were soon identified and grouped into family of four enzymes termed as RSK1-4. It was later on observed that the RSKs may play a minor role in rps6 phosphorylation [1] to give way for the emergence of a major rps6 kinase identified as 70 kDa ribosomal S6 kinase [1–4]. The 70 kDa ribosomal S6 kinase was purified from mitogen stimulated Swiss mouse 3T3 cells [5]. Cloning of 70 kDa ribosomal S6 kinase was soon accomplished after protein sequencing of a rat and rabbit cDNA [6–8]. Subsequent identification and cloning of human orthologs of S6K genes RPS6KB1 and RPS6KB2 encoding S6K1 and S6K2 thus, respectively, led to the emergence of a second family of S6 kinases, a principal kinase of rps6 in somatic cells [2, 9–14].

S6 kinases figure among important signaling molecules required for propagation of cell growth and proliferation. Adding to its importance is the fact that S6 kinase (S6K1) is a downstream effector of mTORC1 (mechanistic target of rapamycin), a master regulator of cell growth and proliferation. Evidences are strong enough to suggest that the mTOR-S6K1 axis controls fundamental cellular processes including transcription, translation and lipid synthesis. Further, this nexus partly regulates glucose homoeostasis, controls insulin sensitivity, adipocyte metabolism, tissue and organ size, learning, memory and aging [15]. S6K1 has therefore been considered to play a pivotal role in regulating cellular physiology. Any dysregulation of this signaling axis has deleterious implications ranging from metabolic disorders to various cancers. mTOR/S6K1 signaling network has hence remained central target for various medical interventions employed over the years for treating the pathological conditions. However, so far the therapeutic interventions involving mTOR as a target molecule for treating cancers, particularly renal carcinomas have reported a fair share of relapses. The resistance shown to these drugs post chronic exposures remain one of the major concerns the scientific community is faced upto. One of the reasons for any such observation would be the incomplete understanding vis-a-vis the regulation of mTOR/S6K1 axis. It is as such conceivable that improved understanding of S6K1 regulation would help develop novel therapeutics that should take care of these challenges. The present chapter reviews the regulation of S6K, its structure and functions.

2. S6K protein family; domain architecture and cellular localization

Based on the sequence alignment of their catalytic domains, S6Ks have been placed in AGC family of protein kinases that mainly comprise of protein kinase A (PKA) protein kinase G (PKG) and protein kinase C (PKC) [16]. The structural resemblance in AGC kinases allows

http://dx.doi.org/10.5772/intechopen.75209

them to exhibit more or less a similar mode of regulation. These kinases fundamentally exhibit bi-lobal structural organization around the kinase domain. This structural arrangement enables their amino and carboxyl termini to coordinate ATP binding [17, 18]. Further these kinases comprise of an activation loop (commonly known as T-loop) that precedes their C-terminus lobe. The phosphorylation of T-loop brings in conformational changes important for phosphoryl transfer. Two other important phosphorylation sites, the turn motif (TM) (so-named due to its location at the cusp of a structural turn in the PKA tail) and hydrophobic motif (HM) sequentially follow the kinase domain. The phosphorylated HM site engages a hydrophobic pocket within the N-lobe. The phosphorylated TM site stabilizes phospho-HM binding to the N-lobe hydrophobic pocket. Together, these three phosphorylations stand critical in stabilizing the catalytically active state [17, 18].

RPS6KB1, human ortholog of S6K1 genes, encodes two isoforms, p70S6K1 and p85S6K1 (formerly known as p70S6KII and p70S6KI, respectively) through alternative translational start sites [10]. p70S6K1, a 502 amino acid protein and its larger isoform p85S6K1, having nuclear localization sequence (NLS) within the 23 amino acid N-terminal extension, were earlier believed to remain localized in the cytoplasm and nucleus, respectively. However, the data generated of late, contests the nuclear localization of p85S6K1 while showing its sub-cellular localization to be cytoplasmic and at the same time claims p70S6K1 localization to be both nuclear and cytoplasmic [19]. S6K2, encoded by RPS6KB2 gene, constitutes

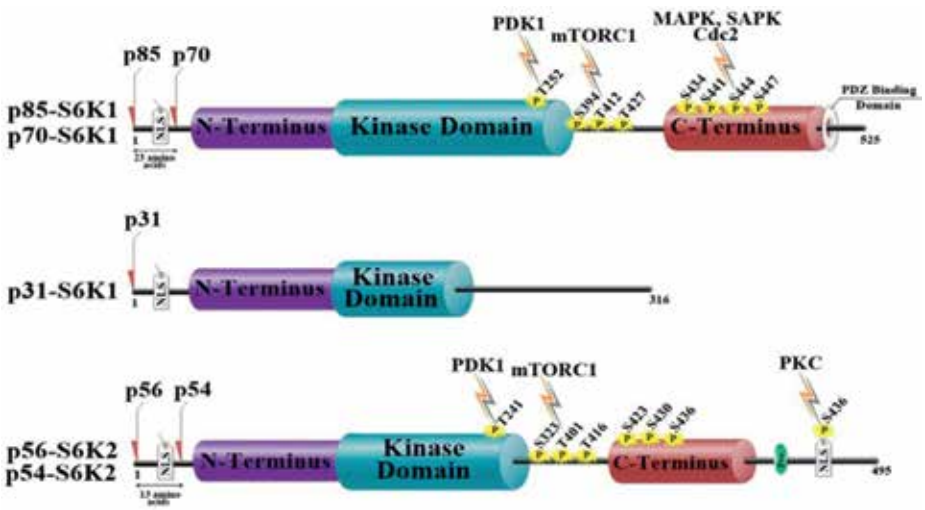

Figure 1. S6K isoforms, structure and domain organization with potential phosphorylating kinases along with their sites of action. S6K1 isoforms include p70-, p85- and p31-S6K1; alternative start site usage lengthens the p85- and p31-S6K1 N-termini by 23 amino acids (note that p31-S6K1 lacks most of the kinase domain). S6K2 isoforms include p54- and p56-S6K2; alternative start site usage lengthens the p56-S6K2 N-terminus by 13 amino acids. NLSs lie within the N-terminal extensions of p85-S6K1 and p56-S6K2, whereas S6K2 additionally contains an NLS within the C-terminus as well as a proline-rich domain (Pro). S6Ks contain an acidic N-terminal domain (NTD), kinase domain (KD), linker region and acidic C-terminal domain (CTD). The N-terminal domain contains the TOS motif, whereas the CTD contains the auto-inhibitory pseudosubstrate domain and RSPRR motif. mTORC1 phosphorylates the HM (hydrophobic motif) site (Thr412) in the linker region and PDK1 phosphorylates the T-loop site (Thr252) within the kinase domain. Other regulatory phosphorylation (P) sites, including the TM (turn motif) site (Ser394), are shown.

another member of S6 kinase family that, via alternative start sites, produces two nuclear isoforms, owing to the presence of nuclear localization sequence at the C-terminus [11]. The longer isoform, p56S6K2 comprises a 13 amino acid extension at N-terminus than its shorter isoform, p54S6K2 [9]. Moreover S6K2 isoforms have been characterized by the presence of proline-rich domain at the C-terminal [20]. S6K1 and S6K2 have been structurally dissected into several regulatory domains, namely an acidic N-terminus that contains the TOR signaling (TOS) motif; the kinase domain that contains the activation/T-loop; a linker region that contains the TM and HM sites; and a basic C-terminus containing an auto-inhibitory pseudosubstrate domain (**Figure 1**). These proteins are homologous to each other within appreciable limits; their catalytic domains share about 83% identity at the amino acid level [9]. However, the differences observed in the extreme N- and C-terminal regions, direct these kinases to distinct compartments or to different molecular targets. Thus, for example, the C-terminal PDZ binding domain in S6K1 allows recruitment to the actin cytoskeleton via binding to neurabin [21] and S6K2 containing a proline-rich region in its C-terminus facilitates interaction with SH3 domain or WW repeat containing protein [9]. Besides full length forms of S6K1 and S6K2, a novel kinase domain truncated splice variant, p31S6K (**Figure 1**) with potential oncogenic properties, has also been reported [22].

3. S6 kinase regulation; a historical perspective for prevalent mode of activation

Research carried out over the past 20 years identified diverse growth factors and mitogens with the ability to activate the S6 kinases [23, 24]. Seemingly among these multiple inputs, insulin/IGF pathway, which signals via phosphoinositide 3-kinase (PI3K) and mTORC1 appears to be the best studied activator of S6K1 [25, 26]. Besides PI3K, other independent signaling pathways like Ras/MAPK (mitogen activated protein kinase) have also been implicated in S6K1 activation [27, 28]. S6K1's contribution in controlling cell growth and proliferation being paramount made the basis for studying its structure and function. The data accumulated over a period of time, hence revealed the molecular insights that govern activation of S6K1 by mitogens and identified, to a large extent, the complex interactions between its specific domains and phosphorylation sites. These observations eventually became the reason for putting forth the models that explain the activation of S6K1 by stepwise multisite phosphorylations [29–33]. These models propose that during inactive state, the basic C-terminal pseudosubstrate domain of S6 K1 interacts with its acidic N-terminus. This interaction obstructs the phosphorylation at kinase domain and renders it inactive [6, 34]. Stimulation by growth factors besides other mitogens result in the phosphorylation of four proline-directed sites at C-terminal auto-inhibitory pseudosubstrate domain (Ser434, Ser441, Ser444 and Ser447) [30, 32]. These phosphorylations induce a conformational change thereby releasing the interaction between the two termini and enables access to the HM and T-loop sites. Phosphorylation at these two critical sites ultimately leads to the full activation of the kinase [6, 34]. Several proline-directed kinases, including ERK1/2, JNK1/2 and CDK1 have been implicated in phosphorylation of these sites; it however remains unclear as to which

kinase(s) play a dominant role in-vivo [32]. Scientific observations during early 1990's propounded the theory that mTOR controls the in-vivo activation of S6K1 [35]. These observations were further augmented during 1998–1999 when mTORC1 was shown to directly phosphorylate the HM site (Thr412) of S6K1 in vitro [36, 37]. The indispensible nature of HM site phosphorylation is further supported by the evidence that mutation of Thr 412 to alanine (T412A) abolishes the S6K1 activity, whereas the phosphor mimicked variant (T412E) enhances basal activity even in absence of mitogens [20, 33, 38]. The findings put forward hence strengthened the notion that phosphorylation of Thr 412 at HM site remains pivotal for S6K1 activation. Co-ordinate phosphorylation of Thr252 in the activation/T-loop and Thr412 at HM site leads to maximal activation of S6K1 [29, 33, 39]. Set out to identify the potential kinases responsible for Thr 252 phosphorylation, in vitro followed by in-vivo studies show PDK1, also corroborated as a kinase for AKT, to directly phosphorylate Thr252 at T-loop site [29, 39, 40]. This was supported by the findings that PDK1 null embryonic stem cells, $PDK1^{-/-}$ or T252A mutation rendered S6K1 enzymatically dead [33, 41]. Further it is observed that PDK1 mediated phosphorylation of C-Terminus truncated variant of S6K1, S6K1-ΔCT remains significantly higher than full length S6K1 while mutation in c-terminal phosphorylation sites to alanine results in poor S6K1 activation [29]. Moreover, PDK1 poorly activates S6K1 T412A-ΔCT or T394A-ΔCT in vitro. All these findings reveal that unphosphorylated c-terminus puts restraint toward full activation of S6K1 by blocking the access of PDK1 to activation loop and the importance of HM and TM sites toward bringing PDK1 mediated S6K1 activation [29]. Phosphorylation of Ser394 (or Ser371 in P70S6K isoform) at turn motif site also remains of much significance for complete activation of S6K1, as S394A substitution of S6K1 renders it completely inactive [42]. Notwithstanding the importance of turn motif site, the data accumulated on regulation and function of this phosphorylation event has progressed, albeit rather slowly. Whatever little the quantum of data may be available for regulation around TM site phosphorylation till date, it concordantly reflects that this site does not represent an autophosphorylation event [42]. Some more interesting observations reveal that even the addition of T412E substitution fails to restore the activity of S394A mutant, thus supporting the notion that this site plays an important yet independent role in regulating the activity of S6K1 [42]. Evidences, though scant, have also emerged which corroborate mTOR in promoting Ser394 phosphorylation in vitro [43]. However, since data accumulated in certain other cases does not correlate phospho-Ser394 well with mTORC1 activity, intermediation of some other kinase besides mTORC1 is also speculated. Reports based on analogy to co-translational phosphorylation of TM site (Thr450) in AKT are tempting to speculate that S6K1 TM site phosphorylation also represents an early event that occurs co-translationally prior to T-loop and HM site phosphorylation [44]. Indeed, a recent report supports such an idea, as Ser394 phosphorylation occurs simultaneously with the production of S6K1 protein from a transfected plasmid [31]. Since the phosphorylations at the HM and T-loop sites exhibit strong cooperativity, the temporal order of these two phosphorylations has not yet been convincingly deciphered. However, the data available till date has put forth two models for S6K1 activation wherein the conventional and widely accepted model suggests mTORC1 mediated phosphorylation at Thr 412 to precede PDK1 mediated phosphorylation at Thr 252 [29, 38, 39]. An alternate model however, suggests that the phosphorylation at T-loop (Thr 252) precedes HM phosphorylation (Thr 412) [31, 33] (**Figure 2**).

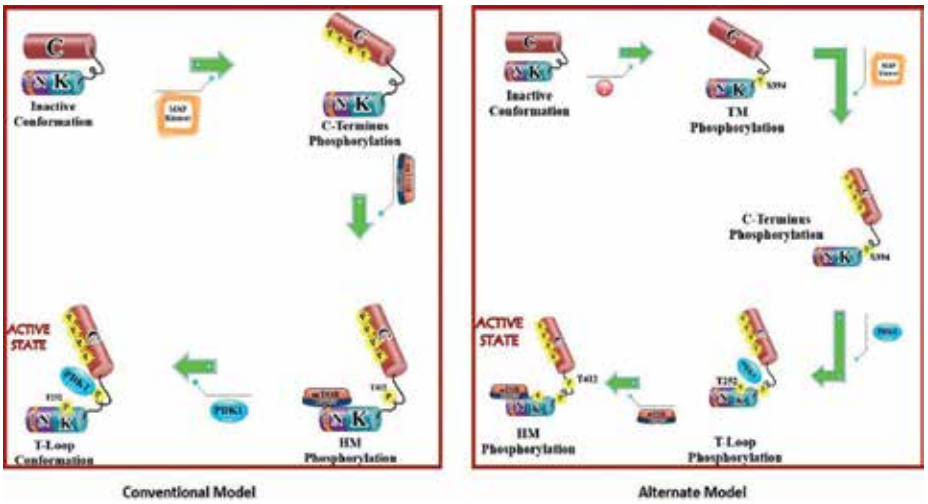

Figure 2. Stepwise activation of S6K1 via multi-site phosphorylation. Conventional model: the interaction of the C- and N-terminal domains results in auto-inhibition of S6K1. Step 1: mitogens promote C-terminal domain (C) phosphorylation on multiple sites to induce a more relaxed conformation. Step 2: the release of the auto-inhibitory C-terminal domain (CTD) enables mTORC1 access to the HM and thus phosphorylation of Thr^{412}. Step 3: the release of the auto-inhibitory CTD and phosphorylation on Thr^{412} enables PDK1-mediated phosphorylation of the T-loop on Thr^{252}, resulting in full activation of S6K1. Phospho-Thr^{412} serves as docking site for PDK1. Owing to insufficient data, the temporal order of TM site phosphorylation (Ser^{394}) is not depicted. Alternative model: Step 1: an unknown kinase phosphorylates the inactive form of S6K on the TM site Ser^{394}. Step 2: mitogens promote C-terminal domain (C) phosphorylation on multiple sites to induce a more relaxed conformation. Step 3: The release of the auto-inhibitory C-terminal domain enables PDK1 access to the T-loop. Step 4: PDK1-mediated phosphorylation of Thr^{252} promotes mTORC1-mediated phosphorylation on the HM site, Thr^{412}. KD, kinase domain; N,N-terminal domain.

S6K1 amino terminus serves a regulatory role in promoting phosphorylation at HM site and thereby at T-loop site that ultimately brings about its complete activation. The regulation by amino terminus is documented to be two pronged. Firstly, it acts as a receptor of an activating input critical for Thr 412 and Thr 252 phosphorylation; and, secondly, it suppresses an inhibitory function mediated by C-terminus. These observations have strong scientific support as the data generated over a period of time shows that amino terminus truncation of S6K1 (ΔNT) abolishes the phosphorylation of rapamycin-sensitive sites Thr 412, Thr 252, Ser 427 and renders it inactive [38, 45] whereas additional deletion of C-terminus (ΔNT/ΔCT) restores all the rapamycin-sensitive phosphorylations as well as the kinase activity of S6K1. The regulatory function of the amino terminus was mapped to a short stretch of amino acids (comprising of 5–9 amino acids and represented by a signature sequence FDIDL) present at extreme end known as TOS motif [20]. It has been shown that S6K1 truncated of TOS motif or its F5A variant (F5A mutation within the FDIDL sequence) abolishes S6K1 kinase activity as well as Thr412 and Thr252 phosphorylation. However, deletion of C-terminus from the F5A mutant (F5A-ΔCT) partially restores kinase activity and Thr 412 phosphorylation [20]. The TOS motif function has been related toward a launch pad required for mTORC1 to engage substrates and to mediate phosphorylation of rapamycin-sensitive sites [46, 47]. The data generated supports the view that mTOR interaction with TOS motif is indirect and

mediated by another member of mTORC1 complex, a scaffold protein called Raptor [46, 47]. For phosphorylation of S6K1 and 4EBP-1, the other mTORC1 effector molecule, binding with raptor has been unequivocally stated to be critical. However, the precise mechanism by which raptor mediates efficient phosphorylation of S6K1 and 4EBP-1 remains largely debatable. Two models have however been proposed to explain this mechanism. The first model suggests that raptor and mTOR associate in two states with varying affinities governed by the nutrient availability. During nutrient starved state Raptor binds tightly to mTOR and renders it inactive. While as the loose-binding complex, formed during nutrient sufficiency, activates mTOR and promotes efficient phosphorylation of mTOR targets [48]. Furthermore, overexpression of raptor increases the amount of mTOR found in the tight-binding complex, thereby explaining the observation that overexpression of raptor inhibits mTOR activity. However, it is interesting to note that rapamycin is able to disrupt the raptor-mTOR interaction regardless of nutrient status [49], but it is phosphate dependent. The second model supports the existence of Raptor as a scaffolding protein for mTORC-1complex, wherein Raptor has been shown to preferentially bind unphosphorylated forms of mTOR targets and recruit the substrates to the mTOR complex for phosphorylation. Role of S6K1 amino terminus in suppressing the inhibitory function of C-terminus remained unclear till identification of an RSPRR motif (a short stretch of 5 amino acids, 433–437) in C-terminus way back in 2005 [47]. This motif has been suggested to negatively regulate S6K1 activation. Evidences, though scant, have propounded that RSPRR motif functions as a docking site for a negative regulator, such as a phosphatase, that is suppressed by mTORC1 [47].R3Amutation of RSPRR motif within the dead ΔNT or TOS motif-mutant (F5A) backbone (NT-R3A or F5A-R3A) has been shown to rescue insulin-stimulated Thr412 phosphorylation and S6K1 activation [15]. These findings though place TOS motif in exhibiting negative control over RSPPR motif inhibition, the exact mechanism behind this regulation still remains largely a mystery.

3.1. Rapamycin, S6K1 inhibition; a chronology of events

In 1970s, a bacterial strain, Streptomyces hygroscopicus was isolated from a soil sample from Easter Island ("Rapa Nui" in the native language) [50]. This strain was found to produce an anti-fungal metabolite [51]. Post purifications, the metabolite was characterized as a macrocyclic lactone and named "Rapamycin" after its birthplace (**Figure 3**) [52].

The strong ability of rapamycin to modulate cellular proliferation besides its promising role in immune suppression for treating various cancers lead to a desperate search in decoding its mode of action and simultaneously initiated hunt for nailing down its targets. The search continued for about two decades and finally culminated in early 1990s with identification of a target protein in yeast, whose mutant conferred resistance to the growth inhibitory effects of rapamycin. The protein was subsequently named as TOR (target of rapamycin) [53–56]. Shortly after, the mammalian counterpart of TOR (mTOR) was identified as physical target of rapamycin by three groups separately in 1994, 1995, respectively [57–59]. However effective mechanism of rapamycin action remained oblivious till intracellular cofactor, the peptidyl-prolyl cis/trans isomerase, immunophilin FK506-binding protein 12 (FKBP12) was shown to bind rapamycin as a gain-of-function component to mediate its inhibitory effect on TOR [56].

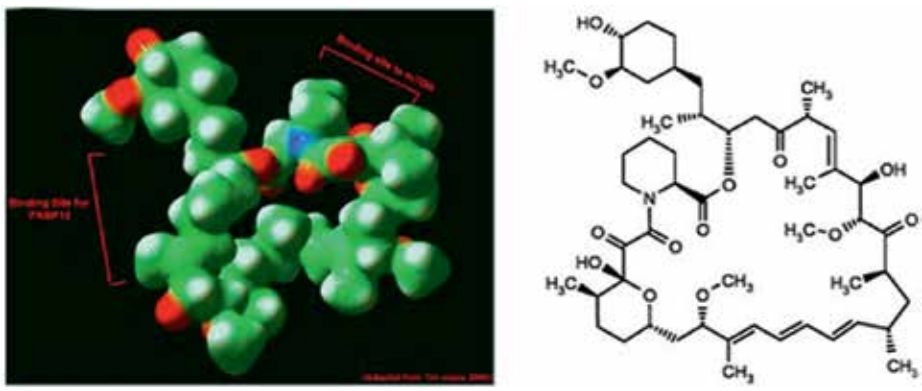

Figure 3. Electron-density model of a molecule of the immunosuppressant drug rapamycin: It is mainly used to prevent rejection in organ transplantation, and is used in kidney transplants. It also has anti-fungal and anti-cancer properties, and was originally derived from soil bacteria found on Easter Island. The chemical formula is C51.H79.N.O13. The atoms are represented here as color-coded blobs: carbon (green), hydrogen (white), nitrogen (blue) and oxygen (red) (adapted from Dr. Tim Evans Science photo library).

Thus, TOR is also referred to as FKBP12 rapamycin associated protein (FRAP) [60].Rapamycin-FKBP12 complex binds to the FKBP12-rapamycin binding (FRB) domain of TOR (**Figure 4**) [55, 60–62] to inhibit its intrinsic kinase activity, including autophosphorylation, thereby inhibiting access of TOR to its substrates [56]. This finding is however in contradiction with the earlier reports wherein it was shown that rapamycin has little effect on mTOR kinase activity [63]. In mammals, rapamycin in complex with FKBP12 acts as an allosteric inhibitor of mTOR complex1 (mTORC1). Only mTORC1 is acutely sensitive to inhibition of rapamycin. However, long term exposure to rapamycin has been shown to inhibit mTORC2 in certain cell types [64]. It is believed that the rapamycin-FKBP12 complex prevents the association between mTOR and raptor; therefore, downstream targets that depend on raptor binding are specifically inhibited [49].

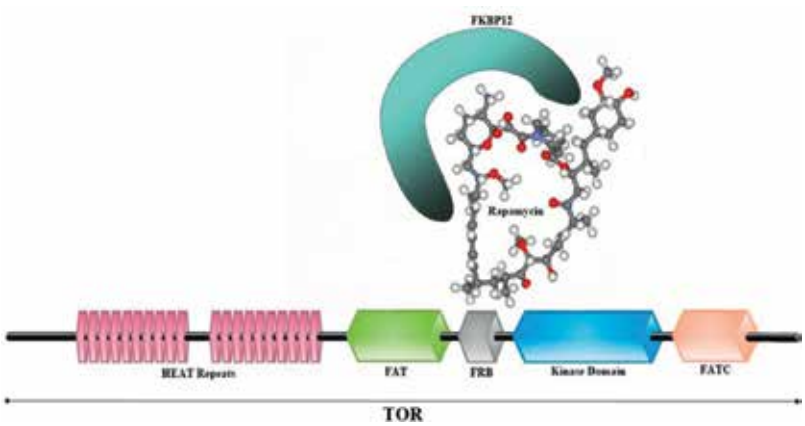

Figure 4. Association of rapamycin with TOR: schematic representation of structural organization of mTOR at domain level and its association with rapamycin–FKBP12 complex.

http://dx.doi.org/10.5772/intechopen.75209

S6K1 being a downstream effector of mTOR, shows inhibitory response to rapamycin as is evidenced by loss of its ability to phosphorylate its substrate, ribosomal protein S6.

Early 1990s witnessed a rigorous search for identifying the rapamycin-sensitive regulatory phosphorylation sites in S6K1 and during 1995 three sites, T252 in the activation/T-loop, T412 in hydrophobic motif and S427 in the linker domain, which connects the auto-inhibitory domain to the catalytic domain were identified as principal rapamycin-sensitive sites [65]. These sites were shown to be dephosphorylated by rapamycin in hierarchical fashion T412 > S427 > T252 with T412 dephosphorylation most closely paralleling loss of kinase activity [65]. Besides, these sites were shown to be responsive to mitogenic stimulation as well [66]. Though all these three sites show rapamycin responsiveness, their conversions to either acidic or neutral amino acids reveal that T252 and T412 were critical regulatory sites where as T427 appeared to play a modulatory function [65]. Further T412 was shown to be principal site of rapamycin-induced S6K1 inactivation as T412E showed increased basal activity and was largely rapamycin resistant [65]. In 1995, Weng et al. reported the cooperativity among the two termini for exhibiting their regulatory effects on S6K1 activation by showing that amino- terminus truncated mutant, S6K1-ΔNT, was inactive. However, additional deletion of carboxy terminus, generating S6K1-ΔNT/ΔCT, rescued its phosphorylation and activation state. Surprisingly it was observed that the mutant was rapamycin resistant [45]. However, its responsiveness toward Wortmannin inhibition did not get compromised. These observations combined with the findings that the carboxy-terminal truncated S6K1 (S6K1-ΔCT) retains rapamycin sensitivity raised the speculations during latter half of 1990's that the inhibitory effect of the rapamycin–FKBP12 complex on S6K1 was either due to blockade of an upstream activator or activation of some phosphatase which mediate their influence through the two termini. The presence of Thr412 phosphorylation on S6K1-ΔNT/ΔCT isolated from rapamycin-treated cells questioned the idea that mTORC1 represents the sole S6K1 Thr412 kinase. In ΔNT/ΔCT, serum and insulin promote Thr412 phosphorylation and kinase activation in a completely rapamycin-resistant manner, suggesting that a rapamycin-insensitive kinase mediates Thr412 phosphorylation [38, 45]. This conundrum was soon resolved in 2005 by observing that rapamycin-insensitive mTORC2 mediates non-physiological S6K1 Thr412 phosphorylation in S6K1 mutants lacking a C-terminus [67]. It was also observed that unlike other AGC kinases S6Ks have atypically an extended C-terminus that imposes additional inhibitory influence on their state of activity [15]. Hence in the dead ΔNT allele, mTORC1 (mTOR/raptor) cannot dock to S6K1 and as such is incapacitated to phosphorylate Thr412, and mTORC2 (mTOR/rictor) cannot phosphorylate Thr412 due to steric hindrance imposed by the extended C-terminus [67]. In the partially rapamycin-resistant ΔCT mutant, both rapamycin-sensitive mTORC1 and rapamycin-insensitive mTORC2 cooperatively mediate Thr412 phosphorylation. In ΔNT/ΔCT (and F5A-ΔCT), only rapamycin-insensitive mTORC2 mediates Thr412 phosphorylation.

4. Other modes of S6K1 regulation

Studies of late propose the involvement of various post translational modifications (PTMs) in regulation of S6K1. Prominent among them are phosphatase-mediated dephosphorylation, acetylation and ubiquitination.

4.1. Dephosphorylation

S6K1 has been proposed to be regulated by co-ordinated action of phosphatases. This was observed while examining the action of rapamycin on phosphorylation state of S6K1.Whereas as rapamycin could dephosphorylate S6K1 through its inhibitory action on mTORC1 complex; the involvement of certain phosphatases to regulate this event was not ruled out. Indeed, S6Ks have been suspected to represent targets of PP2A (protein phosphatase 2A)-like phosphatases. This is supported by the evidence that regulation of several TOR substrates in *S. cerevisiae* occurs via suppression of PP2A-like phosphatases [68]. Further it has also been shown that PP2A interacts with wild type S6K1 and not ΔNT/ΔCT S6K1, a variant truncated of both the termini [69]. It is of late been observed that genetic ablation of PP2A regulatory subunit B (PP2A-B′) in *Drosophila melanogaster* leads to dS6K (Drosophila S6K) deregulation and a variety of metabolic defects [70]. Some further studies report that S6K1 phosphorylation (412) is enhanced in human cells upon knock down of PPP2R5C, the human PP2A-B′ orthologue [70]. All these studies support the role of phosphatases in regulating S6K1 activity. However, it is to be ascertained how mTOR influences these PP2A like phosphatase to modulate S6K1 Thr412 phosphorylation.

4.2. Acetylation and ubiquitination

Acetylation of S6K1 occurs reportedly by the action of two acetyltransferase enzymes, p300/CBP (cAMP-response-element-binding protein binding protein) and PCAF (p300/CBP-associated factor) [71].While as this PTM has been shown to occur on extreme C-terminus of S6K1 in response to the stimulation by mitogens, its dependence on phosphorylation has not been confirmed [72]. Acetylation has been primarily linked to the stabilization of S6K1 [71]. However, the concrete evidence to support this notion has remained largely elusive till date.

Polyubiquitination is another PTM, S6K1 is responsive to. S6K1 appears to experience the ubiquitination in response to mitogen stimulation [73, 74] through direct action of ubiquitin ligase, ROC1 [75]. RNA interference (RNAi) mediated knock down of ROC1 results in stabilization of S6K. These results are hence suggestive of the notion that polyubiquitination causes destabilization of S6K1 and a resultant attenuation of S6K1 downstream signals. Although regulation of S6K1 through ubiquitination still remains in its infancy; it however has opened a new window toward understanding detailed mechanistic inputs of proteasome mediated S6K1 regulation and function.

5. Functions associated with S6K1—a debatable discourse

S6K1, a downstream effector of mTOR is considered to be a multifaceted effector that regulates cell growth and proliferation by phosphorylating multiple ways. To add to its wide range of functions, S6K1 has much recently been shown to play a pivotal role in cellular senescence through its newly found substrate ZRF1 [76]. S6K1 gets activated in response to various signaling pathways including mTOR, PI3-kinase and MAPK, in a coordinated manner through sequential phosphorylation events directed at multiple sites [62]. While S6K1

extends its influence on various cellular functions, some of its associated functions still appear untenable, reason being the inadequate support data. Some of the S6K1 associated functions are briefly described below:

5.1. Cell growth and translational control—what is the connection?

S6K1 has been shown to control cell growth primarily by its ability to phosphorylate S6 protein of 40S ribosomal subunit (rpS6). These phosphorylations are shown to be localized at the C-terminus and happen in sequential order, namely S236 > S235 > S240 > S244 > S247. The dependence of S6 phosphorylation on S6K1 can traced by the observation that rpS6 deficient mice, rps6−/− show phenotypic resemblance to S6K1 deficient mice, S6K1−/−. Although these mice are viable and fertile, however both exhibit cell growth defects. It however is confusing to observe that $S6K1^{-/-}$ mice display minimal defects in rpS6 phosphorylation, while S6 K2, homolog of S6K1, deficient mice, that is, $S6K2^{-/-}$ grow to normal size despite exhibiting a significant reduction in rpS6 phosphorylation [77]. The conundrum can partly be resolved by the observation that claims selective recruitment of S6K1 and S6K2 to different cellular compartments and as such enables them to differentially phosphorylate rpS6 or alternatively by the preposition that rpS6 phosphorylation may ensue at a specific developmental stage where S6K1 but not S6K2 is active. Although a discordance seems to appear vis-a-vis S6K1 activation, rpS6 phosphorylation and cell growth, their complete understanding as far as therapeutics is concerned remains but pivotal. Although S6 phosphorylation was initially thought to be required for selectively translating mRNAs characterized by 5′-oligo Pyrimidine tract (5′-TOP). Later studies however confirmed that 5′ TOP mRNA translation remains unaffected in $S6K1^{-/-}/S6K2^{-/-}$ mice, which display minimal rpS6 phosphorylation [77], hence suggesting these two events to be independent [78].

S6K1 maintains its influence on cellular translation not only by the ability to phosphorylate ribosomal protein S6 (rpS6) but some studies provide evidences to establish S6K1 as a transcription factor kinase as well. One of the study shows S6K1 to phosphorylate eukaryotic initiation factor 4B (eIF4B), a component of cap-binding complex to control cap dependent translation. While as inactive S6K1 associates with eIF3 (a component of pre-initiation complex) and upon activation by mTORC1, via serum stimulation, dissociates to phosphorylate eIF4B, another component of pre-initiation complex [79, 80]. Besides it also controls translational initiation by phosphorylating PDCD4, a negative regulator of eIF4A (**Figure 5**). Phosphorylated PDCD4 gets ultimately degraded by ubiquitin ligase βTRCP [81]. The importance of S6K1 vis-a-vis translational control can further be demonstrated by the observation which implicates S6K1 in controlling translation elongation as well. The study carried shows S6K1 to phosphorylate and inactivate eEF2k (eukaryotic elongation factor-2 kinase, a negative regulator of translation [82].

5.2. Cell cycle progression-a reality check

S6K1 has been extensively discussed as a kinase that drives cells from G1 to S phase during cell cycle progress. However, the data in support does not seem to match this notion completely

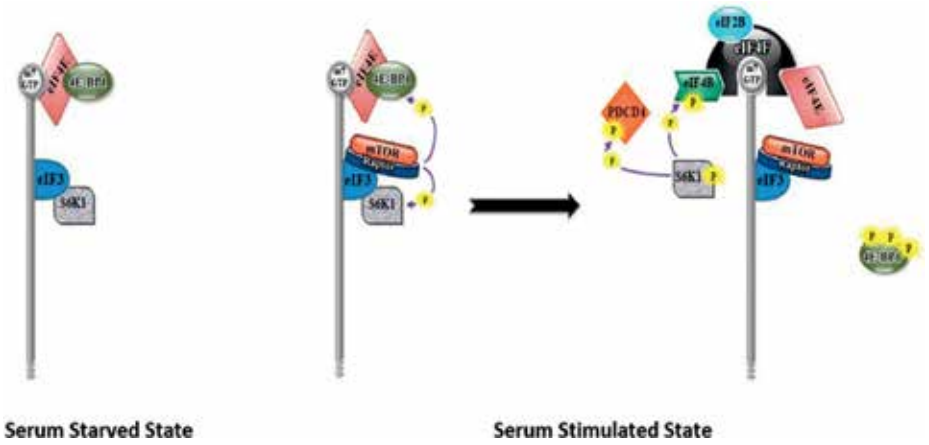

Figure 5. S6K1, a component of translation initiation complex: S6K1 is bound to eIF3 in basal (resting) state cells. Upon serum stimulation, mTORC1 is recruited to the complex, wherein it activates S6K1, which dissociates from eIF3 and phosphorylates several of its targets involved in the initiation of translation.

and in fact some of the data available even contests the veracity of these claims. Whereas Embryonic Stem (ES) cells devoid of S6K1 (S6K1−/−) and mouse embryonic fibroblasts (MEFs) obtained from S6K1 deficient mice do not show significant defects in cell proliferation [77], other studies implicate S6K1 in facilitating cell cycle progression through phosphorylation of estrogen receptor leading to the activation of its target genes that ultimately promotes cellular proliferation [83]. Additionally S6K1 exerts its control over cellular transcription through its ability to phosphorylate cAMP response element binding protein (CREB) isoform CREMr and transcription factor UBF-1 [84]. Phosphorylated UBF-1 in turn activates RNA Polymerase 1 driven transcription of genes encoding ribosomal RNAs and as such aid in ribosomal biogenesis. However, contrary to this belief McMullen et al. proposed no absolute requirement of S6K at either transcriptional or translational levels during ribosomal biogenesis. They showed that $S6K^{-/-}$ mice do not exhibit impaired cardiac hypertrophy, a response dependent on ribosomal biogenesis [85]. These observations put a question mark on the actual involvement of S6K1 in controlling cell cycle progression. Howsoever discordant the views with respect to direct involvement of S6K1 may be but the role of S6K1 in accelerating the cell cycle from G1 to S remains undisputed.

5.3. Cellular metastasis—handy option for therapeutic interventions

S6 kinase, being a downstream effector of mTOR is upregulated in various cancerous situations and as such regulates cellular metastasis. Its involvement in brain tumor pathogenesis was established by the fact that mTOR/S6K axis remains constitutively activated in glioma cells and upon S6K knockdown, the transformed phenotype of these cells is partially rescued [86]. Additionally constitutive activation of S6K1 has been reported in esophageal squamous cell carcinomas (ESCC) [87]. These and several other examples highlight the importance of S6K1 in various cancerous state and as such makes it a potential target for therapeutic interventions to treat cancer. A number of pharmaceutical companies have

http://dx.doi.org/10.5772/intechopen.75209

already ventured in developing S6K specific inhibitors like ATP-competitive compounds that exhibit selective inhibition of S6K. This remains important in the backdrop of cancer relapses/resistance observed while treating them with mTOR inhibitor rapamycin or its rapalogues [88]. However, until recently it was not clear as to what contributes to the rapamycin resistance in these cells. This conundrum was to a greater extent resolved when MAPK interacting kinase (MNK) was found to be an active player in exerting rapamycin resistance in cancer cells. The study deliberated that cancer cell resistance to the mTORC1 inhibitor rapamycin involves MNK activation via a feedback signaling loop elicited by rapamycin [89, 90]. Much recently a study by Brown et al. reveals that post rapamycin treatment mTORC1 activity is sustained by MNK in cancer cells by way of promoting the association between MNK and mTORC1 to form a sub-complex. This way MNK facilitates binding of mTORC1 with its substrates and with phosphatidyl inositol 3′kinase-related kinase (PIKK) stabilizer, TELO2, while discouraging DEPTOR (endogenous mTOR inhibitor) binding [91]. In addition MNKs are also known for phosphorylating eukaryotic initiation factor 4E, eIF4e [92] and as such may play a role in cellular translation. Further the data generated in our lab hints out at the possible role of eIF4e in regulating S6K1 (unpublished data). We believe that prolonged exposure of cells to rapamycin results in activation of MNKs with the resultant activation of eIF4e. As a result S6K1 gets activated to counter the anti-proliferative/anti-cancerous effects of rapamycin (**Figure 6**). Thus therapeutic interventions envisaging S6K1 as a direct target would help overcome, to a larger extent, the resistance experienced toward mTORC1 inhibitors and as such emerge as more appropriate target for treating cancers.

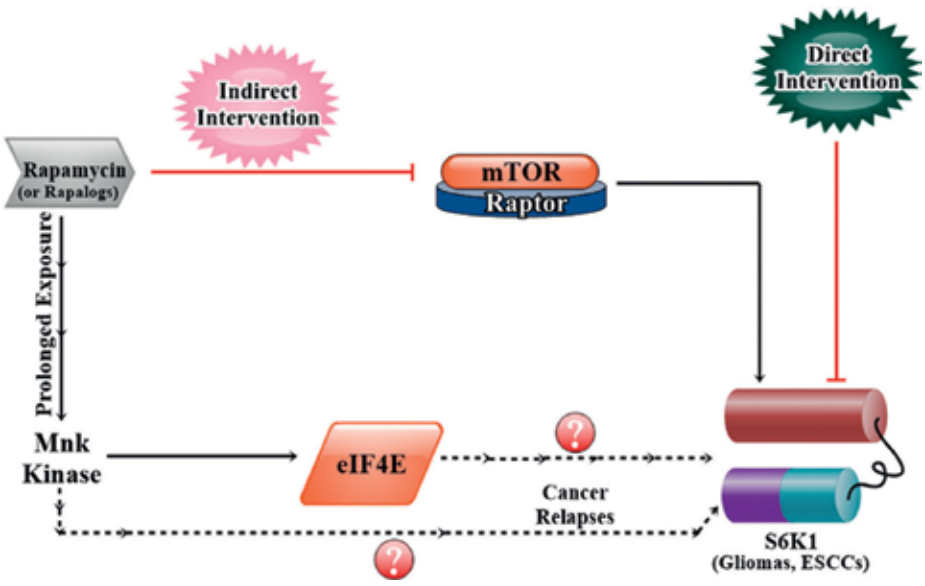

Figure 6. S6K1, a prudent target for treating cancers: an illustration representing S6K1 as a potential target for the specific inhibitors which could be used to overcome the resistance observed while using mTORC1 inhibitors during various cancerous situations.

6. Conclusion

Understanding S6K1 regulation and its associated functions over the last two decades has seen an upward trend. It has now been possible to establish S6K1 an important regulator of cell growth and proliferation. Further our understanding vis-à-vis S6K1 functions have also improved considerably to dissociate many of its functions from the ones related to phosphorylating rpS6 only. Instead its control over various cellular factors makes it more important candidate for regulating cellular physiology. Of late, S6K1 appears as a prudent target for treating various cancers and as such various trials have been initiated to use specific S6K1 inhibitors with the aim to treat different cancers where the routine practices of using generalized drugs appear less effective. However, achieving a complete success in this endeavor still seems difficult. A major challenge in this regard is to ensure that S6K1 s role is tumor specific regardless of its type or origin. Resolving such issues will of course be a task that would attract more research in the years to come.

Conflict of interest

The authors declare that there is no conflict of interest.

Author details

Sheikh Tahir Majeed[1], Rabiya Majeed[1,2], Ghazia Shah[1] and Khurshid I Andrabi[1]*

*Address all correspondence to: andrabik@uok.edu.in

1 Department of Biotechnology, Science block, University of Kashmir, Jammu and Kashmir, India

2 Department of Biochemistry, University of Kashmir, Jammu and Kashmir, India

References

[1] Anjum R, Blenis J. The RSK family of kinases: Emerging roles in cellular signalling. Nature Reviews. Molecular Cell Biology. 2008;**9**(10):747

[2] Chung J, Kuo CJ, Crabtree GR, Blenis J. Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. Cell. 1992;**69**(7): 1227-1236

[3] Price DJ, Calvo V, Avruch J, Bierer BE. Rapamycin-induced inhibition of the 70-kilodalton S6 protein kinase. Science. 1992;**257**(5072):973-977

[4] Stewart MJ, Berry CO, Zilberman F, Thomas G, Kozma SC. The Drosophila p70s6k homolog exhibits conserved regulatory elements and rapamycin sensitivity. Proceedings of the National Academy of Sciences. 1996;**93**(20):10791-10796

[5] Jenö P, Ballou LM, Novak-Hofer I, Thomas G. Identification and characterization of a mitogen-activated S6 kinase. Proceedings of the National Academy of Sciences. 1988;**85**(2):406-410

[6] Banerjee P, Ahmad MF, Grove JR, Kozlosky C, Price DJ, Avruch J. Molecular structure of a major insulin/mitogen-activated 70-kDa S6 protein kinase. Proceedings of the National Academy of Sciences. 1990;**87**(21):8550-8554

[7] Harmann B, Kilimann MW. cDNA encoding a 59 kDa homolog of ribosomal protein S6 kinase from rabbit liver. FEBS Letters. 1990;**273**(1-2):248-252

[8] Kozma SC, Ferrari S, Bassand P, Siegmann M, Totty N, Thomas G. Cloning of the mitogen-activated S6 kinase from rat liver reveals an enzyme of the second messenger subfamily. Proceedings of the National Academy of Sciences. 1990;**87**(19):7365-7369

[9] Gout I, Minami T, Hara K, Tsujishita Y, Filonenko V, Waterfield MD, et al. Molecular cloning and characterization of a novel p70 S6 kinase, p70 S6 kinase β containing a proline-rich region. The Journal of Biological Chemistry. 1998;**273**(46):30061-30064

[10] Grove JR, Banerjee P, Balasubramanyam A, Coffer PJ, Price DJ, Avruch J, et al. Cloning and expression of two human p70 S6 kinase polypeptides differing only at their amino termini. Molecular and Cellular Biology. 1991;**11**(11):5541-5550

[11] Koh H, Jee K, Lee B, Kim J, Kim D, Yun Y-H, et al. Cloning and characterization of a nuclear S6 kinase, S6 kinase-related kinase (SRK); a novel nuclear target of Akt. Oncogene. 1999;**18**(36):5115

[12] Lee-Fruman KK, Kuo CJ, Lippincott J, Terada N, Blenis J. Characterization of S6K2, a novel kinase homologous to S6K1. Oncogene. 1999 Sep;**18**(36):5108-5114

[13] Saitoh M, Ten Dijke P, Miyazono K, Ichijo H. Cloning and characterization of p70S6KβDefines a novel family of p70 S6 kinases. Biochemical and Biophysical Research Communications. 1998;**253**(2):470-476

[14] Shima H, Pende M, Chen Y, Fumagalli S, Thomas G, Kozma SC. Disruption of the p70s6k/p85s6k gene reveals a small mouse phenotype and a new functional S6 kinase. The EMBO Journal. 1998;**17**(22):6649-6659

[15] Magnuson B, Ekim B, Fingar DC. Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks. The Biochemical Journal. 2012;**441**(1):1-21

[16] Hanks SK, Hunter T. Protein kinases 6. The eukaryotic protein kinase superfamily: Kinase (catalytic) domain structure and classification. The FASEB Journal. 1995;**9**(8):576-596

[17] Jacinto E, Lorberg A. TOR regulation of AGC kinases in yeast and mammals. The Biochemical Journal. 2008;**410**(1):19-37

[18] Pearce LR, Komander D, Alessi DR. The nuts and bolts of AGC protein kinases. Nature Reviews. Molecular Cell Biology. 2010;**11**(1):9

[19] Rosner M, Hengstschläger M. Nucleocytoplasmic localization of p70 S6K1, but not of its isoforms p85 and p31, is regulated by TSC2/mTOR. Oncogene. 2011;**30**(44):4509

[20] Schalm SS, Blenis J. Identification of a conserved motif required for mTOR signaling. Current Biology. 2002;**12**(8):632-639

[21] Burnett PE, Blackshaw S, Lai MM, Qureshi IA, Burnett AF, Sabatini DM, et al. Neurabin is a synaptic protein linking p70 S6 kinase and the neuronal cytoskeleton. Proceedings of the National Academy of Sciences. 1998;**95**(14):8351-8356

[22] Karni R, de Stanchina E, Lowe SW, Sinha R, Mu D, Krainer AR. The gene encoding the splicing factor SF2/ASF is a proto-oncogene. Nature Structural & Molecular Biology. 2007;**14**(3):185

[23] Fenton TR, Gout IT. Functions and regulation of the 70 kDa ribosomal S6 kinases. The International Journal of Biochemistry & Cell Biology. 2011;**43**(1):47-59

[24] Meyuhas O, Dreazen A. Ribosomal protein S6 kinase: From TOP mRNAs to cell size. Progress in Molecular Biology and Translational Science. 2009;**90**:109-153

[25] Foster KG, Fingar DC. Mammalian target of rapamycin (mTOR): Conducting the cellular signaling symphony. The Journal of Biological Chemistry. 2010;**285**(19):14071-14077

[26] Zoncu R, Efeyan A, Sabatini DM. mTOR: From growth signal integration to cancer, diabetes and ageing. Nature Reviews. Molecular Cell Biology. 2011;**12**(1):21

[27] Chou MM, Blenis J. The 70 kDa S6 kinase complexes with and is activated by the rho family G proteins Cdc42 and Rac1. Cell. 1996;**85**(4):573-583

[28] Romanelli A, Martin KA, Toker A, Blenis J. p70 S6 kinase is regulated by protein kinase Cζ and participates in a phosphoinositide 3-kinase-regulated signalling complex. Molecular and Cellular Biology. 1999;**19**(4):2921-2928

[29] Alessi DR, Kozlowski MT, Weng Q-P, Morrice N, Avruch J. 3-Phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates and activates the p70 S6 kinase in vivo and in vitro. Current Biology. 1998 Jan 15;**8**(2):69-81

[30] Ferrari S, Bannwarth W, Morley SJ, Totty NF, Thomas G. Activation of p70s6k is associated with phosphorylation of four clustered sites displaying Ser/Thr-Pro motifs. Proceedings of the National Academy of Sciences. 1992;**89**(15):7282-7286

[31] Keshwani MM, Von Daake S, Newton AC, Harris TK, Taylor SS. Hydrophobic motif phosphorylation is not required for activation loop phosphorylation of p70 ribosomal protein S6 kinase 1 (S6K1). The Journal of Biological Chemistry. 2011;**286**(26):23552-23558

[32] Mukhopadhyay NK, Price DJ, Kyriakis JM, Pelech S, Sanghera J, Avruch J. An array of insulin-activated, proline-directed serine/threonine protein kinases phosphorylate the p70 S6 kinase. The Journal of Biological Chemistry. 1992;**267**(5):3325-3335

[33] Weng Q-P, Kozlowski M, Belham C, Zhang A, Comb MJ, Avruch J. Regulation of the p70 S6 kinase by phosphorylation in vivo analysis using site-specific anti-phosphopeptide antibodies. The Journal of Biological Chemistry. 1998;**273**(26):16621-16629

[34] Price DJ, Mukhopadhyay NK, Avruch J. Insulin-activated protein kinases phosphorylate a pseudosubstrate synthetic peptide inhibitor of the p70 S6 kinase. The Journal of Biological Chemistry. 1991;**266**(25):16281-16284

[35] Brown EJ, Beal PA, Keith CT, Chen J, Shin TB, Schreiber SL. Control of p70 s6 kinase by kinase activity of FRAP in vivo. Nature. 1995;**377**(6548):441

[36] Burnett PE, Barrow RK, Cohen NA, Snyder SH, Sabatini DM. RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. Proceedings of the National Academy of Sciences. 1998;**95**(4):1432-1437

[37] Isotani S, Hara K, Tokunaga C, Inoue H, Avruch J, Yonezawa K. Immunopurified mammalian target of rapamycin phosphorylates and activates p70 S6 kinase α in vitro. The Journal of Biological Chemistry. 1999;**274**(48):34493-34498

[38] Dennis PB, Pullen N, Pearson RB, Kozma SC, Thomas G. Phosphorylation sites in the autoinhibitory domain participate in p70s6k activation loop phosphorylation. The Journal of Biological Chemistry. 1998;**273**(24):14845-14852

[39] Pullen N, Dennis PB, Andjelkovic M, Dufner A, Kozma SC, Hemmings BA, et al. Phosphorylation and activation of p70s6k by PDK1. Science. 1998;**279**(5351):707-710

[40] Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PRJ, Reese CB, et al. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Bα. Current Biology. 1997 Apr 1;**7**(4):261-269

[41] Williams MR, Arthur JSC, Balendran A, van der Kaay J, Poli V, Cohen P, et al. The role of 3-phosphoinositide-dependent protein kinase 1 in activating AGC kinases defined in embryonic stem cells. Current Biology. 2000;**10**(8):439-448

[42] Moser BA, Dennis PB, Pullen N, Pearson RB, Williamson NA, Wettenhall RE, et al. Dual requirement for a newly identified phosphorylation site in p70s6k. Molecular and Cellular Biology. 1997;**17**(9):5648-5655

[43] Saitoh M, Pullen N, Brennan P, Cantrell D, Dennis PB, Thomas G. Regulation of an activated S6 kinase 1 variant reveals a novel mammalian target of rapamycin phosphorylation site. The Journal of Biological Chemistry. 2002;**277**(22):20104-20112

[44] Oh WJ, Wu C, Kim SJ, Facchinetti V, Julien L-A, Finlan M, et al. mTORC2 can associate with ribosomes to promote cotranslational phosphorylation and stability of nascent Akt polypeptide. The EMBO Journal. 2010;**29**(23):3939-3951

[45] Cheatham L, Monfar M, Chou MM, Blenis J. Structural and functional analysis of pp70S6k. Proceedings of the National Academy of Sciences. 1995;**92**(25):11696-11700

[46] Nojima H, Tokunaga C, Eguchi S, Oshiro N, Hidayat S, Yoshino K, et al. The mammalian target of rapamycin (mTOR) partner, raptor, binds the mTOR substrates p70 S6 kinase and 4E-BP1 through their TOR signaling (TOS) motif. The Journal of Biological Chemistry. 2003;**278**(18):15461-15464

[47] Schalm SS, Tee AR, Blenis J. Characterization of a conserved C-terminal motif (RSPRR) in ribosomal protein S6 kinase 1 required for its mammalian target of rapamycin-dependent regulation. The Journal of Biological Chemistry. 2005;**280**(12):11101-11106

[48] Lee C-H, Inoki K, Guan K-L. mTOR pathway as a target in tissue hypertrophy. Annual Review of Pharmacology and Toxicology. 2007;**47**:443-467

[49] Kim D-H, Sarbassov DD, Ali SM, King JE, Latek RR, Erdjument-Bromage H, et al. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell. 2002;**110**(2):163-175

[50] Vezina C, Kudelski A, Sehgal SN. Rapamycin (AY-22, 989), a new antifungal antibiotic. Journal of Antibiotics (Tokyo). 1975;**28**(10):721-726

[51] Sehgal SN, Baker H, Vézina C. Rapamycin (AY-22, 989), a new antifungal antibiotic. Journal of Antibiotics (Tokyo). 1975;**28**(10):727-732

[52] Vignot S, Faivre S, Aguirre D, Raymond E. mTOR-targeted therapy of cancer with rapamycin derivatives. Annals of Oncology. 2005;**16**(4):525-537

[53] Heitman J, Movva NR, Hall MN. Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. Science. 1991;**253**(5022):905-909

[54] Kunz J, Henriquez R, Schneider U, Deuter-Reinhard M, Movva NR, Hall MN. Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression. Cell. 1993;**73**(3):585-596

[55] Petroulakis E, Mamane Y, Le Bacquer O, Shahbazian D, Sonenberg N. mTOR signaling: Implications for cancer and anticancer therapy. British Journal of Cancer. 2006;**94**(2):195

[56] Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. Cell. 2006;**124**(3):471-484

[57] Brown EJ, Albers MW, Shin TB, Keith CT, Lane WS, Schreiber SL. A mammalian protein targeted by G1-arresting rapamycin–receptor complex. Nature. 1994;**369**(6483):756

[58] Sabatini DM, Erdjument-Bromage H, Lui M, Tempst P, Snyder SH. RAFT1: A mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. Cell. 1994;**78**(1):35-43

[59] Sabers CJ, Martin MM, Brunn GJ, Williams JM, Dumont FJ, Wiederrecht G, et al. Isolation of a protein target of the FKBP12-rapamycin complex in mammalian cells. The Journal of Biological Chemistry. 1995;**270**(2):815-822

[60] Fingar DC, Blenis J. Target of rapamycin (TOR): An integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. Oncogene. 2004;**23**(18):3151

[61] Gingras A-C, Raught B, Sonenberg N. Regulation of translation initiation by FRAP/mTOR. Genes & Development. 2001;**15**(7):807-826

[62] Schmelzle T, Hall MN. TOR, a central controller of cell growth. Cell. 2000;**103**(2):253-262

[63] Peterson RT, Beal PA, Comb MJ, Schreiber SL. FKBP12-rapamycin-associated protein (FRAP) autophosphorylates at serine 2481 under translationally repressive conditions. The Journal of Biological Chemistry. 2000;**275**(10):7416-7423

[64] Laplante M, Sabatini DM. mTOR signaling. Cold Spring Harbor Perspectives in Biology. 2012;**4**(2):a011593

[65] Pearson RB, Dennis PB, Han JW, Williamson NA, Kozma SC, Wettenhall RE, et al. The principal target of rapamycin-induced p70s6k inactivation is a novel phosphorylation site within a conserved hydrophobic domain. The EMBO Journal. 1995;**14**(21):5279-5287

[66] Han J-W, Pearson RB, Dennis PB, Thomas G. Rapamycin, Wortmannin, and the methylxanthine SQ20006 inactivate p70s6k by inducing dephosphorylation of the same subset of sites. The Journal of Biological Chemistry. 1995;**270**(36):21396-21403

[67] Ali SM, Sabatini DM. Structure of S6 kinase 1 determines whether raptor-mTOR or rictor-mTOR phosphorylates its hydrophobic motif site. The Journal of Biological Chemistry. 2005;**280**(20):19445-19448

[68] Düvel K, Broach JR. The role of phosphatases in TOR signaling in yeast. In: TOR. Springer; 2004. p. 19-38. ISBN 978-3-642-18930-2

[69] Peterson RT, Desai BN, Hardwick JS, Schreiber SL. Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FKBP12–rapamycin associated protein. Proceedings of the National Academy of Sciences. 1999;**96**(8):4438-4442

[70] Hahn K, Miranda M, Francis VA, Vendrell J, Zorzano A, Teleman AA. PP2A regulatory subunit PP2A-B′ counteracts S6K phosphorylation. Cell Metabolism. 2010;**11**(5):438-444

[71] Fenton TR, Gwalter J, Ericsson J, Gout IT. Histone acetyltransferases interact with and acetylate p70 ribosomal S6 kinases in vitro and in vivo. The International Journal of Biochemistry & Cell Biology. 2010;**42**(2):359-366

[72] Fenton TR, Gwalter J, Cramer R, Gout IT. S6K1 is acetylated at lysine 516 in response to growth factor stimulation. Biochemical and Biophysical Research Communications. 2010;**398**(3):400-405

[73] Gwalter J, Wang M-L, Gout I. The ubiquitination of ribosomal S6 kinases is independent from the mitogen-induced phosphorylation/activation of the kinase. The International Journal of Biochemistry & Cell Biology. 2009;**41**(4):828-833

[74] Wang M-L, Panasyuk G, Gwalter J, Nemazanyy I, Fenton T, Filonenko V, et al. Regulation of ribosomal protein S6 kinases by ubiquitination. Biochemical and Biophysical Research Communications. 2008;**369**(2):382-387

[75] Panasyuk G, Nemazanyy I, Filonenko V, Gout I. Ribosomal protein S6 kinase 1 interacts with and is ubiquitinated by ubiquitin ligase ROC1. Biochemical and Biophysical Research Communications. 2008;**369**(2):339-343

[76] Barilari M, Bonfils G, Treins C, Koka V, De Villeneuve D, Fabrega S, et al. ZRF1 is a novel S6 kinase substrate that drives the senescence programme. The EMBO Journal. 2017;**36**(6):736-750

[77] Pende M, Um SH, Mieulet V, Sticker M, Goss VL, Mestan J, et al. S6K1−/−/S6K2−/− mice exhibit perinatal lethality and rapamycin-sensitive 5′-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. Molecular and Cellular Biology. 2004;**24**(8):3112-3124

[78] Ruvinsky I, Sharon N, Lerer T, Cohen H, Stolovich-Rain M, Nir T, et al. Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis. Genes & Development. 2005;**19**(18):2199-2211

[79] Holz MK, Ballif BA, Gygi SP, Blenis J. mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. Cell. 2005;**123**(4):569-580

[80] Raught B, Peiretti F, Gingras A-C, Livingstone M, Shahbazian D, Mayeur GL, et al. Phosphorylation of eucaryotic translation initiation factor 4B Ser422 is modulated by S6 kinases. The EMBO Journal. 2004;**23**(8):1761-1769

[81] Dorrello NV, Peschiaroli A, Guardavaccaro D, Colburn NH, Sherman NE, Pagano M. S6K1-and \s sTRCP-mediated degradation of PDCD4 promotes protein translation and cell growth. Science. 2006;**314**(5798):467-471

[82] Wang X, Li W, Williams M, Terada N, Alessi DR, Proud CG. Regulation of elongation factor 2 kinase by p90RSK1 and p70 S6 kinase. The EMBO Journal. 2001;**20**(16):4370-4379

[83] Yamnik RL, Digilova A, Davis DC, Brodt ZN, Murphy CJ, Holz MK. S6 kinase 1 regulates estrogen receptor α in control of breast cancer cell proliferation. The Journal of Biological Chemistry. 2009;**284**(10):6361-6369

[84] Hannan KM, Thomas G, Pearson RB. Activation of S6K1 (p70 ribosomal protein S6 kinase 1) requires an initial calcium-dependent priming event involving formation of a high-molecular-mass signalling complex. The Biochemical Journal. 2003;**370**(2):469-477

[85] McMullen JR, Shioi T, Zhang L, Tarnavski O, Sherwood MC, Dorfman AL, et al. Deletion of ribosomal S6 kinases does not attenuate pathological, physiological, or insulin-like growth factor 1 receptor-phosphoinositide 3-kinase-induced cardiac hypertrophy. Molecular and Cellular Biology. 2004;**24**(14):6231-6240

[86] Nakamura JL, Garcia E, Pieper RO. S6K1 plays a key role in glial transformation. Cancer Research. 2008;**68**(16):6516-6523

[87] Hou G, Xue L, Lu Z, Fan T, Tian F, Xue Y. An activated mTOR/p70S6K signaling pathway in esophageal squamous cell carcinoma cell lines and inhibition of the pathway by rapamycin and siRNA against mTOR. Cancer Letters. 2007;**253**(2):236-248

[88] Wilkinson JE, Burmeister L, Brooks SV, Chan C-C, Friedline S, Harrison DE, et al. Rapamycin slows aging in mice. Aging Cell. 2012;**11**(4):675-682

[89] Carracedo A, Ma L, Teruya-Feldstein J, Rojo F, Salmena L, Alimonti A, et al. Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. The Journal of Clinical Investigation. 2008;**118**(9):3065-3074

[90] Wang X, Yue P, Chan C-B, Ye K, Ueda T, Watanabe-Fukunaga R, et al. Inhibition of mammalian target of rapamycin induces phosphatidylinositol 3-kinase-dependent and Mnk-mediated eukaryotic translation initiation factor 4E phosphorylation. Molecular and Cellular Biology. 2007;**27**(21):7405-7413

[91] Brown MC, Gromeier M. MNK controls mTORC1: Substrate association through regulation of TELO2 binding with mTORC1. Cell Reports. 2017;**18**(6):1444-1457

[92] Pyronnet S, Imataka H, Gingras A-C, Fukunaga R, Hunter T, Sonenberg N. Human eukaryotic translation initiation factor 4G (eIF4G) recruits mnk1 to phosphorylate eIF4E. The EMBO Journal. 1999;**18**(1):270-279

Edited by Fernanda Lasakosvitsch and Sergio Dos Anjos Garnes

The human body is composed of several systems and organs, consisting of millions of cells that need relatively stable conditions to function and contribute to the survival of the body as a whole. The maintenance of stable conditions for the cells against the variations of the external environment is an essential function of the body and is called homeostasis. As a consequence of the loss of homeostasis, a disease is manifested. This book aims to provide the reader with an up-to-date view of the self-regulatory mechanisms that are activated to achieve homeostasis, the pathways that are altered during the disease process, and how medicine can intervene to restore balance in critical patients.

Published in London, UK

IntechOpen

ISBN 978-1-83881-606-3