Research Article

Tocotrienol-Rich Fraction (TRF) Treatment Promotes Proliferation Capacity of Stress-Induced Premature Senescence Myoblasts and Modulates the Renewal of Satellite Cells: Microarray Analysis

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Human skeletal muscle is a vital organ involved in movement and force generation. It suffers from deterioration in mass, strength, and regenerative capacity in sarcopenia. Skeletal muscle satellite cells are involved in the regeneration process in response to muscle loss. Tocotrienol, an isomer of vitamin E, was reported to have a protective effect on cellular aging. This research is aimed at determining the modulation of tocotrienol-rich fraction (TRF) on the gene expressions of stress-induced premature senescence (SIPS) human skeletal muscle myoblasts (CHQ5B). CHQ5B cells were divided into three groups, i.e., untreated young control, SIPS control (treated with 1 mM hydrogen peroxide), and TRF-posttreated groups (24 hours of 50 μg/mL TRF treatment after SIPS induction). The differential gene expressions were assessed using microarray, GSEA, and KEGG pathway analysis. Results showed that TRF treatment significantly regulated the gene expressions, i.e., p53 (RRM2B, SESN1), ErbB (EREG, SHC1, and SHC3), and FoxO (MSTN, SMAD3) signalling pathways in the SIPS myoblasts compared to the SIPS control group ($p < 0.05$). TRF treatment modulated the proliferation capacity of SIPS myoblasts through regulation of ErbB (upregulation of expression of *EREG*, *SHC1*, and *SHC3*) and FoxO (downregulation of expression of *MSTN* and *SMAD3*) and maintaining the renewal of satellite cells through p53 signalling (upregulation of *RRM2B* and *SESN1*), MRF, cell cycle, and Wnt signalling pathways.

1. Introduction

Skeletal muscle is one of the largest organs in the body and contributed to 45-55% of the total body weight [1]. However, skeletal muscle cells are not able to self-replenish as it is a terminally differentiated cells. Thus, skeletal muscle cells require a population of resident adult stem cells, satellite cells for maintenance and repair [2]. Satellite cells are typically mitotically quiescent in resting muscle and will be activated to prepare for cell cycle entry upon stimulation and during muscle injury. A subset of satellite cells will be self-renewed to maintain the satellite cell pool and regenerate a rapidly proliferating transit-amplifying myoblast population.

Regeneration of skeletal muscle cells deteriorated with the aging process. This muscle degeneration process due to aging is termed sarcopenia [3]. Muscle degeneration started with 0.5–1% after 30 years of age and the rate increases as age reaches 65 years [4]. The mechanism of this degeneration is still yet to be deciphered clearly, but it can involve oxidative stress [5]. Muscle atrophy is reported to be stimulated by the PI3K/Akt and NF-κB signalling pathways [6]. Activation of PI3K/Akt signalling regulates the skeletal muscle mass and

metabolism in skeletal muscle [7]. On the other hand, inhibition of this signalling pathway would inhibit the FoxO protein and upregulate the atrophy-related gene (atrogin), such as atrogin1/MAFbx1 and MuRF [7, 8]. Braun and Gautel proposed that NF-κB also would involve in the regulation of atrogin and regulate the catabolism and anabolism of muscle protein [7].

Myostatin, a member of the TGF superfamily, was reported as a muscle atrophy inducer by activating the transcription factor SMAD3 which leads to atrogin-1 expression and inhibition of Akt/mTOR signalling and protein synthesis. Moreover, SMAD3 is also involved in the inhibition of peroxisome proliferator-activated receptor-coactivator-1 (PGC1) promoter activity and increased FoxO-mediated signalling [9]. Besides the mentioned FoxO signalling pathway and the expression of atrogin in muscle atrophy, Brack et al. suggested a different signalling pathway in muscle aging, which is the activation of the Wnt/β-catenin signalling pathway that leads to increased muscle fibrosis [10].

Cell senescence is categorised into three major mechanisms, i.e., replicative senescence, oncogene-induced senescence, and stress-induced premature senescence (SIPS) [11]. SIPS cells and replicative senescence cells are similar in molecular action and morphology, such as flat and larger cells, increased activities of senescence-associated β-galactosidase, and cell cycle arrest. However, SIPS was not affected by the length of telomere [12–14]. Hydrogen peroxide (H_2O_2) is a common stress mediator in the SIPS model as it induced the cells to have senescence morphology alike with the replicative senescence cells [11, 12]. Generation of H_2O_2 by the mitochondria or external sources from the cells leads to the damage of mitochondrial components and thus initiated the degeneration process [15].

Since the degeneration of skeletal muscle was closely related to oxidative stress, it was suggested that reestablishment of the redox balance would be beneficial in the amelioration of age-related degeneration in skeletal muscle [16]. Vitamin E, especially tocotrienol, has been widely reported for its antioxidant effects in preventing aging [17–19]. Supplementation of vitamin E (α-tocopherol) together with vitamin C reduced oxidative stress and increased the activities of antioxidant enzymes in the rat skeletal muscle [20].

Various approaches, including pharmacological interventions, nutrition, and physical activities, have been reported to improve the aging conditions of the skeletal muscle. However, to date there is neither supplementation nor intervention that works effectively with minimum side effects [3]. Howard et al. reported that tocopherol promoted the repair of plasma membrane in mouse myocytes [21]. However, these findings were focused on the effects of tocopherol, but not tocotrienol, on skeletal muscle. Previously, our study revealed that the tocotrienol-rich fraction (TRF) has reduced the senescence morphology and improved the proliferation capacity towards stress-induced premature senescence cells [22]. Hence, we would like to identify and elucidate the mechanism involved in the regenerative action of TRF on stress-induced premature skeletal muscle cells.

2. Materials and Methods

2.1. CHQ5B Cell Culture.
Human satellite cells were isolated from a biopsy of a 5-day-old infant quadriceps muscle and were kindly provided by Dr. Vincent Mouly from UMRS 787, Institut de Myologie, INSERM, Université Pierre et Marie Curie, Paris, France. Upon isolation, the satellite cells proliferated in culture as myoblasts (known as CHQ5B cells) and were considered to be at 1 mean population doubling (MPD). CHQ5B cells were cultivated in a growth medium at 37°C in a humid atmosphere containing 5% carbon dioxide (CO_2) as described previously [22].

2.2. Treatment Protocols.
The Tocotrienol-rich fraction (TRF) was purchased from Sime Darby Sdn. Bhd., Selangor, Malaysia (TRF Gold Tri E 70). TRF consists of α-tocotrienol (26.67%), β-tocotrienol (4.29%), γ-tocotrienol (32.60%), δ-tocotrienol (15.53%), and α-tocopherol (20.81%) [22]. The CHQ5B cells (PD 29 ± 3) were incubated with different treatments, i.e., untreated young control, stress-induced premature senescence (SIPS), and TRF-treated group (SIPS myoblasts posttreated with TRF). Untreated young control cells were cultured without any treatment with ROS inducer or TRF. The SIPS model was established by exposure of CHQ5B cells to the stressor, 1 mM H_2O_2 diluted in growth medium for 30 minutes [22, 23]. In the TRF-treated group, the CHQ5B cells were exposed to the stressor at 1 mM H_2O_2 for 30 minutes followed by incubation in 50 μg/mL TRF (Sime Darby Bioganic Sdn Bhd) for 24 hours [22]. This concentration and duration of TRF treatment were in line with a previous study which showed that SIPS cells posttreated with 24 hours 50 μg/mL TRF were able to increase the proliferation capacity of myoblast cells.

2.3. Total RNA Extraction and Purification.
Total RNA from CHQ5B cells in different treatment groups was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instruction. Polyacryl Carrier (Molecular Research Center) was added in each extraction to precipitate the total RNA. The extracted total RNA pellet was then washed with 75% ethanol and air-dried. The extracted total RNA was dissolved in RNase and DNase-free distilled water and purified by using the RNeasy® Mini Kit (Qiagen, USA) according to the manufacturer's instruction. Total RNA was then stored at −80°C immediately after extraction and purification. RNA concentration and purity of the extracted RNA were determined by NanoDrop (Thermo Scientific, USA). The quality of RNA was assessed by an Agilent 2100 bioanalyzer (Agilent Technologies, USA). The high quality of RNA, i.e., RNA integrity number (RIN) ranging from 7 to 10 and absorbance ratio of A260 to A280 ranging from 1.5 to 2.1, was utilized for microarray analysis (Figure S01, Supplementary Materials).

2.4. Gene Expression Microarray Profiling.
The isolated RNA was amplified and labelled using Affymetrix GeneChip 3' IVT Express and Affymetrix GeneChip Hybridisation, Wash, and Stain Kit (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's protocol. The RNA was then hybridised to the Affymetrix GeneChip PrimeView Human Gene Expression Array cartridge, washed, and scanned according

to the manufacturer's protocol. The arrays from three samples each of untreated young control, SIPS control, and TRF-treated cells were scanned and processed by using the AGCC Scan Control (Affymetrix).

2.5. Gene Expression Microarray Data Analysis and Statistics. The raw CEL data files from microarray profiling were imported into the Partek Genomics Suite (v. 6.6; Partek, St. Louis, MO) for analysis, and two-way analysis of variance (2-way ANOVA) was applied with a fold change of 1.5 for the selection of differentially expressed genes at a significance level of $p < 0.05$. The differentially expressed gene lists were further correlated for their relevant biological function and reaction pathway by analysing the GSEA (Gene Set Enrichment Analysis) and KEGG (Kyoto Encyclopedia of Genes and Genomes) using the Partek Genomic Suite. A significance level of $p < 0.05$ in the GSEA analysis to identify the significant biological process involved was observed, whereas an enrichment score of $p < 0.05$ in the KEGG pathway to identify the significant pathway was observed.

2.6. Quantitative Real-Time PCR (qPCR). The microarray data was validated by using qualitative qPCR. Genes for validation, i.e., GDF15, EREG, RRM2B, SHC3, SHC1, SESN1, MSTN, MYOD1, and SMAD3, were chosen from pathway analysis. By using 2 μL total RNA as template and iScript Reverse Transcription Supermix (Bio-Rad, USA), the cDNA is generated from RNA. The reactions were carried out as follows: priming for 5 minutes at 25°C, then reverse-transcription for 30 minutes at 42°C and inactivation of the reverse-transcription for 5 min at 85°C.

Primer sequences for GDF15, EREG, RRM2B, SHC3, SHC1, SESN1, MSTN, MYOD1, and SMAD3 are shown in Table 1(a). qPCR was carried out by using 1 μL cDNA as template, 1 μL of forward and reverse primers for genes of interest, and SSoAdvanced SYBR Green Supermix (Bio-Rad, USA). All reactions were run in duplicate using Real-Time PCR iQ5 (Bio-Rad, USA). The thermal cycling profiles are presented in Table 1(b). The melt curve analysis of each pair of primers and agarose gel electrophoresis that was performed on the PCR products was used to determine the primer specificity (Figure S02, Supplementary Materials). The gene expression level of each targeted gene was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). It was presented as relative expression value (REV) by using the $2^{-\Delta\Delta Ct}$ method of relative quantification and the following equation:

$$\text{REV} = 2^{\text{Ct value of GAPDH} - \text{Ct value of the gene of interest}}. \quad (1)$$

By referring to each REV value of the targeted gene, the fold change (FC) can be calculated by using the following equation:

$$\text{Fold change} = \text{REV}_{\text{treated cells}} / \text{REV}_{\text{untreated cells}}. \quad (2)$$

2.7. Statistical Analysis. Microarray data was analysed by using Partek Genomic Suite (v. 6.6; Partek, St. Louis, MO), and the differentially expressed gene lists were filtered based

on a fold change of 1.5 and a significance level of $p < 0.05$ by using two-way analysis of variance (2-way ANOVA). The relevant biological function and reaction pathway was identified based on GSEA analysis at a significance level of $p < 0.05$ and KEGG analysis at an enrichment score $p < 0.05$ by using the Partek Genomic Suite.

The REV data in qPCR are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed with the software IBM SPSS Statistics (version 20). Independent sample T test was used to determine the significant differences in between the SIPS control and TRF-treated groups. For all of the tests, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Quality Control Assessment of the Samples and the Hierarchical Clustering of Significantly Expressed Genes. Principal component analysis (PCA) is a multivariate statistic which allows viewing of separation between groups of replicates. The untreated young control, SIPS, and TRF-posttreated groups were well separated (Figure 1(a)). Hierarchical cluster analysis was performed to organize genes into cluster based on their similarities of expression. The upregulation of gene expression was indicated in red, whereas the downregulation of gene expression was indicated in blue. Clustering analysis was able to distinguish gene expressions between untreated young control and SIPS groups as well as between TRF-posttreated and SIPS groups (Figure 1(b)).

3.2. Identification of Gene Expression Changes Associated with SIPS Myoblasts. The gene expression analysis using Partek Genomic Suite was performed to identify changes in the SIPS myoblasts. Statistical analysis of two-way analysis of variance (2-way ANOVA) revealed that a total of 41 genes were significantly regulated in SIPS myoblasts as compared to untreated young control cells (fold change < −1.5 or fold c hange > 1.5; $p < 0.05$); i.e., 11 genes were upregulated and 30 genes were downregulated (Figure 1(c)). The complete list of 41 differentially expressed genes is available in Table S01, Supplementary Materials.

3.3. Identification of Gene Expression Changes Associated with TRF-Post-treatment on SIPS Myoblasts. The gene expression analysis using Partek Genomic Suite was performed to identify changes in TRF-posttreated SIPS myoblasts. Statistical analysis of two-way analysis of variance (2-way ANOVA) revealed that a total of 905 genes were significantly regulated in TRF-posttreated SIPS myoblasts as compared to the SIPS group (fold change < −1.5 or fold change > 1.5; $p < 0.05$); i.e., 378 genes were upregulated and 527 genes were downregulated (Figure 1(c)). The complete list of 905 differentially expressed genes is available in Table S02, Supporting Materials. At present, only selected differentially expressed genes including growth differentiation factor 15 (*GDF15*), epiregulin (*EREG*), ribonucleotide reductase M2B (*RRM2B*), SHC (Src homology 2 domain containing) transforming protein 3 (*SHC3*), SHC transforming protein 1 (*SHC1*), sestrin 1 (*SESN1*), myostatin (MSTN), myogenic differentiation 1 (*MYOD1*), and SMAD

Table 1: Quantitative real-time PCR: (a) primer sequences and (b) the thermal cycling profile.

(a)

Accession number	Gene	Primer type	Primer sequences (5′–3′)
NM_001289746	GAPDH	Forward	TTGCCCTCAACGACCACTTT
		Reverse	TGGTCCAGGGGTCTTACTCC
NM_004864.2	GDF15	Forward	CAGGACGGTGAATGGCTCTC
		Reverse	TAGCGTTTCCGCAACTCTCG
NM_001432.2	EREG	Forward	CTGCAGGTGTGAAGTGGGTT
		Reverse	GTGGAACCGACGACTGTGAT
NM_015713.4	RRM2B	Forward	AGAGTTCTCGCCGGTTTGTC
		Reverse	GTCGACCTCTTCTGCTGTCC
NM_016848.5	SHC3	Forward	GCTTTTGCTGTGAGCAGCC
		Reverse	CCACCGTTAAAAGCCAGCAC
NM_183001.4	SHC1	Forward	CTTGGGAGCTACATTGCCTGT
		Reverse	TCAAAAAGCTCTCTGCCTGGAC
NM_014454.2	SESN1	Forward	TTGACAGCTCCACAACGTGA
		Reverse	TGTACACGAAAGGGCAGTCT
NM_005259.2	MSTN	Forward	TTGACATGAACCCAGGCACT
		Reverse	GTCCTGGGAAGGTTACAGCA
NM_002478.4	MYOD1	Forward	CGCCAGGATATGGAGCTACT
		Reverse	GAGTGCTCTTCGGGTTTCAG
NM_005902.3	SMAD3	Forward	CATGTCGTCCATCCTGCCTT
		Reverse	TTGGTGTTGACGTTCTGCGT

(b)

Cycling step	Temperature	Time	Number of cycles
Enzyme activation/initial DNA denaturation	95°C	30 seconds	1
Denaturation	95°C	5 seconds	40
Annealing/extension	60°C	30 seconds	
Melt curve	65°C to 95°C (in 0.5°C increments)	5 seconds	1

family member 3 (SMAD3) that have attracted our interest will be discussed. The chosen genes regulated by TRF treatment were selected based on the biological processes and KEGG pathway analysis.

3.4. Biological Processes and Pathways Affected by SIPS. GSEA analysis was carried out by using the Partek Genomic Suite. GSEA analysis revealed the selected significant biological processes involved in SIPS myoblasts compared to the untreated young control group ($p < 0.05$) (Table 2). The positive value of the normalized enrichment score (NES) indicated an increment in the regulation of the biological process, whereas the negative value of NES indicated a reduction in the regulation of the stated biological process. SIPS myoblasts showed a significant increase in cellular biogenic amine metabolic process and apoptosis but decreased in activities of lipoprotein and regulation of skeletal muscle cell differentiation.

*3.5. Biological Processes and Pathways Affected by the TRF-Post-treatment on SIPS Myoblasts.* GSEA analysis

revealed the selected significant biological processes involved in response to TRF-posttreatment SIPS myoblasts compared to SIPS control myoblasts ($p < 0.05$) (Table 3). TRF treatment has significantly increased the regulation of JNK cascade, cell growth, and adult walking behavior, but decreased the cell cycle activity. KEGG pathway analysis was carried out by using Partek Genomic Suite, and the data can be categorised according to the cluster of differentially expressed genes (fold change < -1.5 or fold change > 1.5; $p < 0.05$) and the pathway ANOVA statistical method ($p < 0.05$).

Our KEGG pathway analysis showed that TRF treatment significantly modulated the p53 signalling pathway, FoxO signalling pathway, Wnt signalling pathway, cell cycle, and ErbB signalling pathway (Table 4). The regulation of gene expression involved in the stated pathway is presented in Figure 2.

3.6. Microarray Result Validation. To confirm the microarray expression, we performed qPCR validation and found that all of the tested genes have consistent gene profiles. The REV
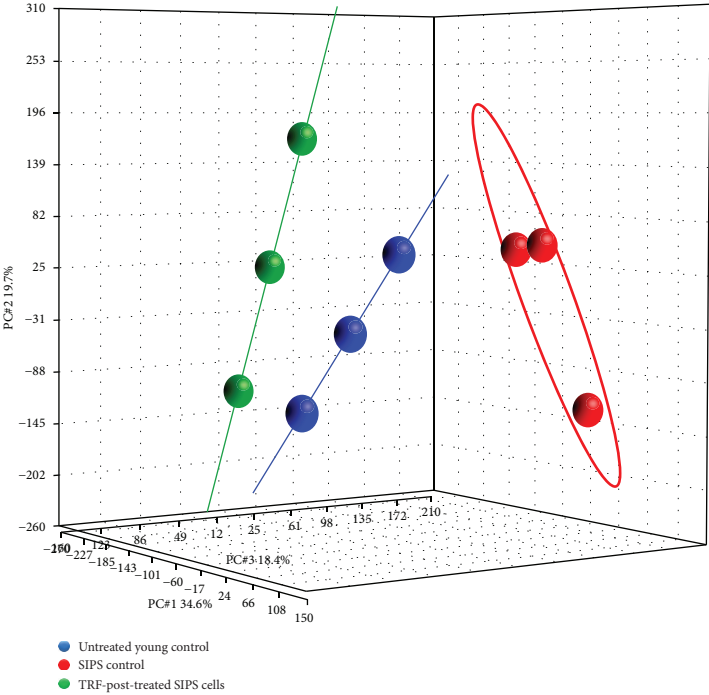
(a)
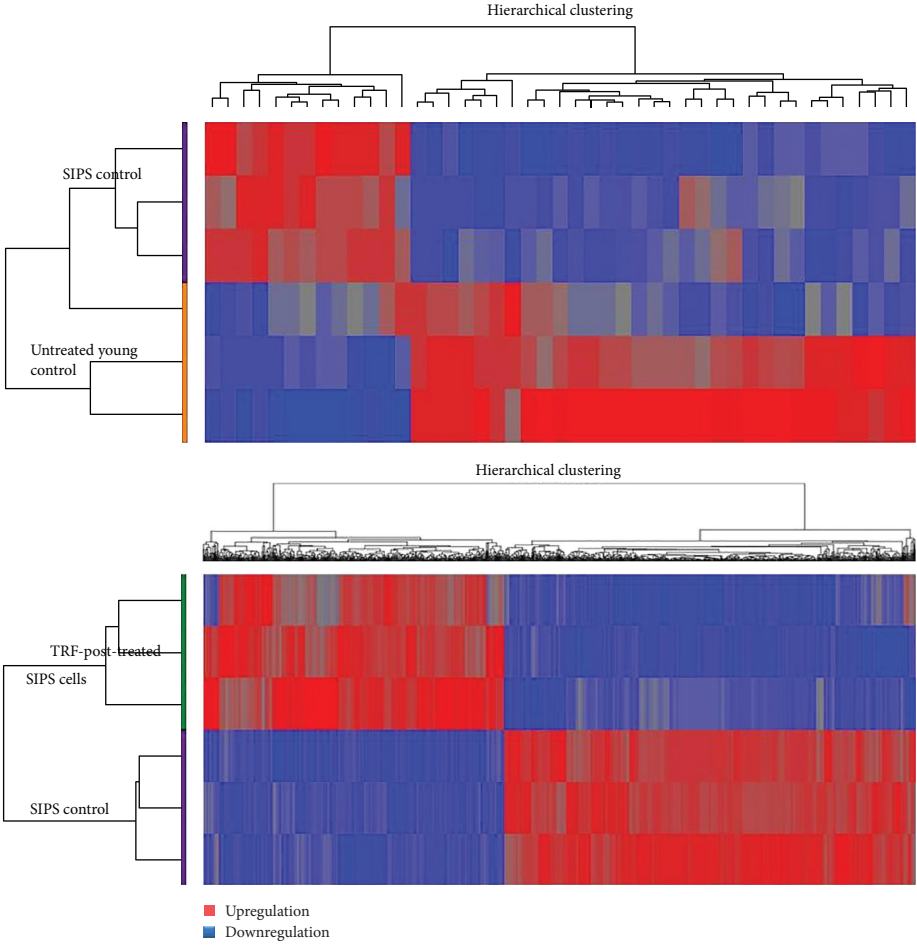
(b)

FIGURE 1: Continued.

Treatment Group / Gene Expression	SIPS control vs Untreated young control	TRF-post-treated SIPS cells vs SIPS control
Upregulation	11	378
Downregulation	30	527
Total	41	905

(c)

FIGURE 1: (a) PCA and (b) hierarchical clustering of the data. Clustering analysis was able to distinguish gene expression between untreated young control and SIPS control as well as between the TRF-treated group and the SIPS control group. (c) There were a total of 41 genes and 905 genes significantly expressed in between SIPS control and untreated young control and in between TRF-posttreated SIPS cells and SIPS control, respectively.

value and fold change for all of the genes were consistent for both microarray analysis and qPCR analysis (Figure 3).

4. Discussion

Vitamin E, especially tocopherol, with a combination of other approaches, such as exercise, vitamin C, or selenium, has been reported to improve the condition of aged skeletal muscles by reducing oxidative stress and increasing the activities of antioxidant enzymes in the muscles of aged rats [20, 24]. Recently, one of the isomers of vitamin E, i.e., tocotrienol, has been proven for its effects in antioxidant defence enhancement and for improving the proliferation and differentiation in both replicative senescence and oxidative stress-induced premature myoblasts [22, 25, 26]. Khor et al. suggested that TRF reduced the senescence phenotypes in the skeletal muscle which may not only be limited to the encounter of oxidative stress but also instead be possibly associated with its regenerative capacity [23]. Little is known on the mechanism of tocotrienol towards the oxidative stress-associated senescence in myoblasts, especially human myoblasts. Thus, in this study, we revealed the novel insight into the differential gene expression by stress-induced human myoblasts with the treatment of TRF.

4.1. Alteration in Gene Expression and Biological Process in SIPS Cells.
Hydrogen peroxide (H_2O_2) is a common inducer in the SIPS model in various cells [27, 28]. Research has shown that H_2O_2 downregulated the expression of miRNAs such as miR-15 and miR-106b families which contributes to some of the features of senescence cells. These features include the increased resistance to apoptosis and activation of p21^{CDKN1A}. At the same time, it upregulates miR-182 which causes specific changes in senescence-associated gene expression [29]. Our GSEA analysis showed that SIPS has upregulated the cellular biogenic amine metabolic process in the cells while differential gene expression analysis showed that spermine oxidase (SMOX) was upregulated. Biogenic amine, such as polyamine, monoamine, and histamine, is involved in the mechanism of apoptosis progression [30]. The increasing expression of SMOX would increase the oxidation of spermine in the nucleus to spermidine, H_2O_2, and 3-aminopropanal and reduce the concentration of nuclear spermine, thus dysregulating the protective roles of spermine in free radical scavenging and DNA shielding and resulting in an overall increased potential for oxidative DNA damage in these cells [31]. Apoptosis has always been one of the

suggested factors in the mass loss of skeletal muscle and muscle atrophy [32, 33], and our GSEA analysis was in line with this factor. The GSEA analysis showed the upregulation of cytochrome c released from mitochondria which is an indicator of early-stage apoptosis in SIPS myoblasts.

4.2. Alteration in Gene Expression, Biological Process, and Signalling Pathway in TRF-Post-treated SIPS Cells

4.2.1. ErbB Signalling Pathway.
KEGG pathway analysis showed that TRF treatment on SIPS myoblasts significantly regulated a few biological processes and pathways related to cell proliferation, including the ErbB signalling pathway and FoxO signalling pathway. Several ErbB signalling-related genes, such as EGF, EGFR, EREG, SHC1, and SHC3, were significantly regulated by the treatment of TRF. The upregulation of SHC1 and SHC3 expression by 2.70-fold and 2.36-fold, respectively, by TRF treatment on SIPS myoblasts suggested the involvement of TRF in modulating the ErbB pathway, hence improving the regenerative capacity of skeletal muscle via cell proliferation. A similar effect was observed in C. elegans, in which SHC1 activated JNK signalling by binding to the MEK-1 kinase and hence regulating stress response and aging [34]. Epiregulin (EREG), an ErbB-signalling related gene, was upregulated with a 5.68-fold in TRF-posttreatment SIPS myoblasts compared to the SIPS group. Upregulation of EREG expression was reported before in C2C12 mouse myoblast cells after 4 hours from differentiation of myoblast cells. However, downregulation of the MRF gene and regulation of canonical Wnt signalling by the TRF on SIPS myoblasts minimised the possibilities of differentiation of human myoblasts (CHQ5B) in this study. Other studies with human cells showed that upregulation of EREG expression improved the proliferation ability of various human cells, i.e., mesenchymal stem cells, keratinocytes, and fibroblasts [35–37]. Hence, upregulation of EREG expression by TRF-post-treatment in SIPS myoblasts suggested a similar mechanism, i.e., modulation of the ErbB pathway which leads to increased proliferation capacity of myoblasts.

4.2.2. Myostatin (MSTN).
Unpredictably, our findings showed that myostatin (MSTN), a negative regulator in the muscle growth and regenerative potential of skeletal muscle, was downregulated significantly by 5.46-fold. This would be a novel finding in the modulation of muscle regeneration by TRF. Exposure of myostatin to mouse C2C12 myoblast

TABLE 2: GSEA analysis on the biological processes regulated by the stress-induced premature senescence as compared to the untreated young control ($p < 0.05$).

GO accession	Biological process	NES	p value
GO:0006576	Cellular biogenic amine metabolic process	1.33	$2.63E - 02$
GO:0044106	Cellular amine metabolic process	1.33	$2.63E - 02$
GO:0046885	Regulation of hormone biosynthetic process	−1.40	$3.17E - 02$
GO:0051004	Regulation of lipoprotein lipase activity	−1.32	$3.28E - 02$
GO:0070874	Negative regulation of glycogen metabolic process	−1.44	$3.39E - 02$
GO:0032106	Positive regulation of response to extracellular stimulus	−1.48	$3.45E - 02$
GO:0032109	Positive regulation of response to nutrient levels	−1.48	$3.45E - 02$
GO:0031442	Positive regulation of mRNA 3′-end processing	−1.24	$3.57E - 02$
GO:0043001	Golgi to plasma membrane protein transport	−1.41	$3.64E - 02$
GO:2000973	Regulation of pro-B cell differentiation	−1.69	$3.77E - 02$
GO:0032494	Response to peptidoglycan	1.40	$3.85E - 02$
GO:0006400	tRNA modification	1.50	$3.92E - 02$
GO:0045807	Positive regulation of endocytosis	−1.34	$4.11E - 02$
GO:0006970	Response to osmotic stress	−1.15	$4.17E - 02$
GO:0090199	Regulation of release of cytochrome c from mitochondria	1.29	$4.35E - 02$
GO:0042953	Lipoprotein transport	−1.18	$4.35E - 02$
GO:0044872	Lipoprotein localization	−1.18	$4.35E - 02$
GO:0061418	Regulation of transcription from RNA polymerase II promoter in response to hypoxia	−1.52	$4.35E - 02$
GO:0048644	Muscle organ morphogenesis	−1.70	$4.35E - 02$
GO:0022615	Protein to membrane docking	−1.49	$4.41E - 02$
GO:0035563	Positive regulation of chromatin binding	1.55	$4.44E - 02$
GO:0048259	Regulation of receptor-mediated endocytosis	−1.25	$4.48E - 02$
GO:0032922	Circadian regulation of gene expression	−1.25	$4.55E - 02$
GO:0006694	Steroid biosynthetic process	−1.37	$4.55E - 02$
GO:2001014	Regulation of skeletal muscle cell differentiation	−1.57	$4.55E - 02$
GO:2000641	Regulation of early endosome to late endosome transport	−1.31	$4.62E - 02$
GO:0043112	Receptor metabolic process	−1.25	$4.69E - 02$
GO:0006891	Intra-Golgi vesicle-mediated transport	−1.38	$4.69E - 02$
GO:0035330	Regulation of hippo signalling	−1.44	$4.69E - 02$
GO:0035640	Exploration behavior	−1.47	$4.69E - 02$
GO:0006270	DNA replication initiation	1.46	$4.76E - 02$
GO:0031536	Positive regulation of exit from mitosis	1.29	$4.76E - 02$
GO:0048020	CCR chemokine receptor binding	−1.41	$4.76E - 02$
GO:0017144	Drug metabolic process	−1.42	$4.76E - 02$
GO:2000095	Regulation of Wnt signalling pathway, planar cell polarity pathway	−1.69	$4.76E - 02$
GO:0042347	Negative regulation of NF-kappaB import into nucleus	−1.37	$4.84E - 02$
GO:0046854	Phosphatidylinositol phosphorylation	−1.38	$4.84E - 02$
GO:0006901	Vesicle coating	−1.42	$4.84E - 02$
GO:0051045	Negative regulation of membrane protein ectodomain proteolysis	1.62	$4.88E - 02$
GO:0006335	DNA replication-dependent nucleosome assembly	1.51	$4.88E - 02$
GO:0034723	DNA replication-dependent nucleosome organization	1.51	$4.88E - 02$
GO:2000649	Regulation of sodium ion transmembrane transporter activity	−1.33	$4.92E - 02$
GO:0048260	Positive regulation of receptor-mediated endocytosis	−1.35	$4.92E - 02$
GO:0002381	Immunoglobulin production involved in immunoglobulin-mediated immune response	−1.36	$4.92E - 02$
GO:0018879	Biphenyl metabolic process	−1.45	$4.92E - 02$

Table 3: GSEA analysis on the biological processes regulated by the TRF-posttreated SIPS cells as compared to the SIPS control ($p < 0.05$).

GO accession	Biological process	NES	p value
GO:0009165	Nucleotide biosynthetic process	−1.36	1.85E − 02
GO:0006338	Chromatin remodeling	−1.76	1.85E − 02
GO:0046328	Regulation of JNK cascade	1.21	1.96E − 02
GO:0030968	Endoplasmic reticulum unfolded protein response	1.71	2.04E − 02
GO:0032069	Regulation of nuclease activity	1.65	2.04E − 02
GO:0016558	Protein import into peroxisome matrix	1.41	2.13E − 02
GO:0030259	Lipid glycosylation	1.56	2.22E − 02
GO:0090501	RNA phosphodiester bond hydrolysis	−1.64	2.22E − 02
GO:0071695	Anatomical structure maturation	1.39	2.27E − 02
GO:0032869	Cellular response to insulin stimulus	1.35	2.27E − 02
GO:0016049	Cell growth	1.55	2.38E − 02
GO:0019321	Pentose metabolic process	1.44	2.44E − 02
GO:2001241	Positive regulation of the extrinsic apoptotic signalling pathway in absence of ligand	1.29	2.44E − 02
GO:2001239	Regulation of the extrinsic apoptotic signalling pathway in the absence of ligand	1.24	2.44E − 02
GO:0032092	Positive regulation of protein binding	1.64	2.50E − 02
GO:0045599	Negative regulation of fat cell differentiation	1.29	2.56E − 02
GO:0071479	Cellular response to ionizing radiation	−1.37	3.17E − 02
GO:0006308	DNA catabolic process	−1.56	3.28E − 02
GO:0000082	G1/S transition of mitotic cell cycle	−1.77	3.28E − 02
GO:0044843	Cell cycle G1/S phase transition	−1.77	3.28E − 02
GO:0090025	Regulation of monocyte chemotaxis	1.52	3.57E − 02
GO:0046325	Negative regulation of glucose import	1.34	3.57E − 02
GO:0010800	Positive regulation of peptidyl-threonine phosphorylation	−1.28	3.57E − 02
GO:0007127	Meiosis I	−1.66	3.64E − 02
GO:0002689	Negative regulation of leukocyte chemotaxis	1.81	3.70E − 02
GO:0006534	Cysteine metabolic process	1.32	3.70E − 02
GO:0090231	Regulation of spindle checkpoint	−1.61	3.70E − 02
GO:1902850	Microtubule cytoskeleton organization involved in mitosis	−1.73	3.70E − 02
GO:0032200	Telomere organization	−1.88	3.70E − 02
GO:0071300	Cellular response to retinoic acid	1.26	3.77E − 02
GO:0006302	Double-strand break repair	−1.88	3.77E − 02
GO:0031064	Negative regulation of histone deacetylation	1.47	3.85E − 02
GO:0010155	Regulation of proton transport	1.37	3.85E − 02
GO:0032924	Activin receptor signalling pathway	−1.35	3.85E − 02
GO:0051181	Cofactor transport	1.62	3.92E − 02
GO:0000060	Protein import into nucleus, translocation	−1.71	3.92E − 02
GO:1902668	Negative regulation of axon guidance	1.67	4.00E − 02
GO:0043388	Positive regulation of DNA binding	1.39	4.00E − 02
GO:0043320	Natural killer cell degranulation	−1.34	4.00E − 02
GO:0010887	Negative regulation of cholesterol storage	1.62	4.08E − 02
GO:0032845	Negative regulation of homeostatic process	1.47	4.08E − 02
GO:0051896	Regulation of protein kinase B signalling	1.33	4.08E − 02
GO:0008334	Histone mRNA metabolic process	−1.69	4.08E − 02
GO:0097191	Extrinsic apoptotic signalling pathway	1.22	4.17E − 02
GO:0002686	Negative regulation of leukocyte migration	1.53	4.26E − 02
GO:0048706	Embryonic skeletal system development	1.36	4.26E − 02
GO:0097035	Regulation of membrane lipid distribution	1.35	4.26E − 02
GO:0061157	mRNA destabilization	−1.72	4.26E − 02

TABLE 3: Continued.

GO accession	Biological process	NES	p value
GO:2000615	Regulation of histone H3-K9 acetylation	−1.73	4.26E − 02
GO:0072528	Pyrimidine-containing compound biosynthetic process	−1.88	4.26E − 02
GO:1903036	Positive regulation of response to wounding	1.35	4.35E − 02
GO:1900746	Regulation of vascular endothelial growth factor signalling pathway	−1.53	4.35E − 02
GO:0009303	rRNA transcription	−1.45	4.41E − 02
GO:0045080	Positive regulation of chemokine biosynthetic process	1.88	4.44E − 02
GO:0008207	C21-steroid hormone metabolic process	1.69	4.44E − 02
GO:0050795	Regulation of behavior	1.51	4.44E − 02
GO:1902107	Positive regulation of leukocyte differentiation	1.28	4.44E − 02
GO:0017157	Regulation of exocytosis	1.16	4.44E − 02
GO:0007098	Centrosome cycle	−1.42	4.44E − 02
GO:0001946	Lymphangiogenesis	−1.43	4.48E − 02
GO:0050919	Negative chemotaxis	1.68	4.55E − 02
GO:0060338	Regulation of the type I interferon-mediated signalling pathway	1.65	4.55E − 02
GO:0050748	Negative regulation of the lipoprotein metabolic process	1.56	4.55E − 02
GO:0038031	Noncanonical Wnt signalling pathway via JNK cascade	1.53	4.55E − 02
GO:0010935	Regulation of macrophage cytokine production	1.49	4.55E − 02
GO:0060907	Positive regulation of macrophage cytokine production	1.40	4.55E − 02
GO:0034661	ncRNA catabolic process	−1.71	4.55E − 02
GO:0090503	RNA phosphodiester bond hydrolysis, exonucleolytic	−1.59	4.62E − 02
GO:1902624	Positive regulation of neutrophil migration	1.69	4.65E − 02
GO:2000406	Positive regulation of T cell migration	1.63	4.65E − 02
GO:2000403	Positive regulation of lymphocyte migration	1.56	4.65E − 02
GO:1901725	Regulation of histone deacetylase activity	2.14	4.76E − 02
GO:1902667	Regulation of axon guidance	1.81	4.76E − 02
GO:0048841	Regulation of axon extension involved in axon guidance	1.73	4.76E − 02
GO:0050882	Voluntary musculoskeletal movement	1.51	4.76E − 02
GO:0042113	B cell activation	−1.19	4.76E − 02
GO:0072522	Purine-containing compound biosynthetic process	−1.29	4.76E − 02
GO:1901991	Negative regulation of mitotic cell cycle phase transition	−1.47	4.76E − 02
GO:0006919	Activation of cysteine-type endopeptidase activity involved in apoptotic process	−1.23	4.84E − 02
GO:2000757	Negative regulation of peptidyl-lysine acetylation	−1.80	4.84E − 02
GO:0035067	Negative regulation of histone acetylation	−1.83	4.84E − 02
GO:0007628	Adult walking behavior	1.94	4.88E − 02
GO:0021756	Striatum development	−1.31	4.92E − 02
GO:1901988	Negative regulation of cell cycle phase transition	−1.46	4.92E − 02
GO:0045185	Maintenance of protein location	−1.48	4.92E − 02
GO:0051053	Negative regulation of DNA metabolic process	−1.52	4.92E − 02
GO:0051258	Protein polymerization	−1.53	4.92E − 02
GO:0044774	Mitotic DNA integrity checkpoint	−1.68	4.92E − 02
GO:0009124	Nucleoside monophosphate biosynthetic process	−1.71	4.92E − 02
GO:0043487	Regulation of RNA stability	−1.75	4.92E − 02
GO:0034501	Protein localization to kinetochore	−1.82	4.92E − 02
GO:0000077	DNA damage checkpoint	−1.91	4.92E − 02
GO:0000079	Regulation of cyclin-dependent protein serine/threonine kinase activity	−2.01	4.92E − 02

cells reduced the proliferation and protein synthesis and thus prevented the progression of cells from the G1 to S phase in the cell cycle [38, 39]. On the other hand, aside from an increment in total RNA and protein synthesis rate, mice with myostatin knockout had a larger size of myotube [40]. A study showed that muscle-associated senescence upregulated

TABLE 4: KEGG pathway analysis regulated by the TRF-posttreated SIPS cells as compared to the SIPS control ($p < 0.05$) according to the (a) differential expression of gene and (b) pathway ANOVA analysis.

(a)

KEGG entry	Pathway	NES	p value
hsa04110	Cell cycle	25.93	$5.50E - 12$
hsa03030	DNA replication	20.99	$7.64E - 10$
hsa04115	p53 signalling pathway	16.76	$5.25E - 08$
hsa05206	MicroRNAs in cancer	16.08	$1.03E - 07$
hsa03460	Fanconi anemia pathway	14.93	$3.29E - 07$
hsa05203	Viral carcinogenesis	14.45	$5.30E - 07$
hsa05200	Pathways in cancer	12.88	$2.54E - 06$
hsa03430	Mismatch repair	10.17	$3.81E - 05$
hsa05322	Systemic lupus erythematosus	10.00	$4.53E - 05$
hsa04114	Oocyte meiosis	9.11	$1.11E - 04$
hsa05034	Alcoholism	8.64	$1.76E - 04$
hsa03420	Nucleotide excision repair	7.96	$3.50E - 04$
hsa03440	Homologous recombination	6.78	$1.13E - 03$
hsa00900	Terpenoid backbone biosynthesis	6.72	$1.21E - 03$
hsa05219	Bladder cancer	6.38	$1.70E - 03$
hsa05166	HTLV-I infection	6.16	$2.11E - 03$
hsa00310	Lysine degradation	6.02	$2.43E - 03$
hsa05210	Colorectal cancer	5.56	$3.86E - 03$
hsa04914	Progesterone-mediated oocyte maturation	5.20	$5.52E - 03$
hsa04068	FoxO signalling pathway	5.10	$6.10E - 03$
hsa05205	Proteoglycans in cancer	4.75	$8.61E - 03$
hsa04978	Mineral absorption	4.50	$1.11E - 02$
hsa04390	Hippo signalling pathway	4.49	$1.12E - 02$
hsa05222	Small cell lung cancer	4.23	$1.46E - 02$
hsa05217	Basal cell carcinoma	4.06	$1.72E - 02$
hsa05212	Pancreatic cancer	4.04	$1.76E - 02$
hsa00480	Glutathione metabolism	3.40	$3.34E - 02$
hsa05214	Glioma	3.17	$4.22E - 02$
hsa04310	Wnt signalling pathway	3.16	$4.25E - 02$

(b)

KEGG entry	Pathway	p value	Fold change
hsa05323	Rheumatoid arthritis	$8.98E - 03$	1.13
hsa04630	Jak-STAT signalling pathway	$1.12E - 02$	1.07
hsa04978	Mineral absorption	$1.56E - 02$	1.13
hsa00860	Porphyrin and chlorophyll metabolism	$1.89E - 02$	1.14
hsa04340	Hedgehog signalling pathway	$1.93E - 02$	1.10
hsa00520	Amino sugar and nucleotide sugar metabolism	$2.06E - 02$	1.11
hsa05217	Basal cell carcinoma	$2.12E - 02$	1.10
hsa00561	Glycerolipid metabolism	$2.18E - 02$	1.09
hsa00630	Glyoxylate and dicarboxylate metabolism	$2.20E - 02$	-1.16
hsa04330	Notch signalling pathway	$2.22E - 02$	1.08
hsa00564	Glycerophospholipid metabolism	$2.33E - 02$	1.07
hsa00100	Steroid biosynthesis	$2.46E - 02$	-1.20
hsa00040	Pentose and glucuronate interconversions	$2.66E - 02$	1.16

TABLE 4: Continued.

KEGG entry	Pathway	p value	Fold change
hsa00051	Fructose and mannose metabolism	2.76E − 02	1.10
hsa03050	Proteasome	2.79E − 01	1.11
hsa00980	Metabolism of xenobiotics by cytochrome P450	3.08E − 02	1.08
hsa00650	Butanoate metabolism	3.11E − 02	−1.13
hsa00052	Galactose metabolism	3.13E − 02	1.12
hsa04640	Hematopoeitic cell lineage	3.22E − 02	1.08
hsa03060	Protein export	3.34E − 02	1.13
hsa04130	SNARE interactions in vesicular transport	3.35E − 02	1.08
hsa04110	Cell cycle	3.39E − 02	−1.31
hsa04966	Collecting duct acid secretion	3.48E − 02	1.23
hsa03430	Mismatch repair	3.68E − 02	−1.38
hsa03030	DNA replication	3.70E − 02	−1.80
hsa03460	Fanconi anemia pathway	3.79E − 02	−1.29
hsa00533	Glycosaminoglycan biosynthesis-keratan sulfate	3.99E − 02	1.12
hsa04012	ErbB signalling pathway	4.04E − 02	1.05
hsa03450	Nonhomologous end-joining	4.25E − 02	−1.19
hsa04070	Phosphatidylinositol signalling system	4.28E − 02	1.06
hsa00190	Oxidative phosphorylation	4.33E − 02	1.08
hsa00730	Thiamine metabolism	4.41E − 02	1.17
hsa03440	Homologous recombination	4.43E − 02	−1.16
hsa00982	Drug metabolism - cytochrome P450	4.55E − 02	1.04
hsa00072	Synthesis and degradation of ketone bodies	4.65E − 02	−1.27
hsa00532	Glycosaminoglycan biosynthesis - chondroitin sulfate/dermatan sulfate	4.69E − 02	1.15
hsa04360	Axon guidance	4.74E − 02	1.05
hsa00280	Valine, leucine, and isoleucine degradation	4.93E − 02	−1.07
hsa04210	Apoptosis	4.96E − 02	1.06
hsa00300	Lysine biosynthesis	4.99E − 02	−1.47

the expression of myostatin, but antagonists of myostatin activated the satellite cells and increased the protein level of Pax7 and MyoD which in turn improved the regenerative capacity of muscle cells [41, 42].

The signalling pathways for myostatin can be divided into SMAD-mediated and non-SMAD pathways. The present microarray analysis showed that the treatment of TRF on SIPS myoblasts modulated the FoxO signalling pathway through downregulation of *MSTN* and *SMAD3*. FoxO and SMAD are involved in the regulation of muscle growth through amplification of atrophy response and activation of MSTN expression [43]. SMAD3-mediated myostatin signalling increased the sarcomeric protein degradation via the ubiquitin-proteasome pathway by stimulating FoxO1 and atrogin-1 expression [44]. In this study, the downregulation of *MSTN* and *SMAD3* by TRF treatment suggested that the reduction in the SMAD-mediated myostatin signalling pathway and TRF treatment would reduce the protein degradation in the SIPS myoblasts.

4.2.3. Ribonucleotide Reductase M2B (RRM2B). Modulation of the ErbB signalling pathway and the FoxO signalling pathway by TRF treatment suggested that the mechanism involved might be related to cell proliferation. However, at

the same time KEGG analysis demonstrated that TRF regulated the satellite cells to remain in the quiescent state through the p53 signalling, cell cycle, and Wnt signalling pathways. Interestingly, these findings showed a contradiction. Nevertheless, recent studies showed that the satellite cells are a heterogeneous population as they consist of satellite stem cells and committed progenitors [45]. Symmetric division of satellite cells allowed the cells to divide and expand the satellite stem cell subpopulation, whereas asymmetric division of satellite cells would maintain the stem cell population which is involved in the self-renewal and generation of myogenic progenitors [46, 47]. Dumont et al. suggested that a dynamic balance must exist in between the symmetric and asymmetric division even though the satellite cells are able to choose whether to perform symmetric or asymmetric division, and it allows them to coordinate their activity with the needs of the regenerating muscle [46]. An imbalance in the ratio of symmetric and asymmetric division would deteriorate the regenerative capacity of muscles. These were observed in the aging cells with a disruption in the satellite cells' ability to self-renew or return to the quiescent state [48, 49].

The TRF-post-treatment on SIPS myoblasts has targeted p53 signalling by upregulating the expression of the p53 target gene, i.e., *RRM2B* and *SESN1*. *RRM2B* activation is

(a)

(b)
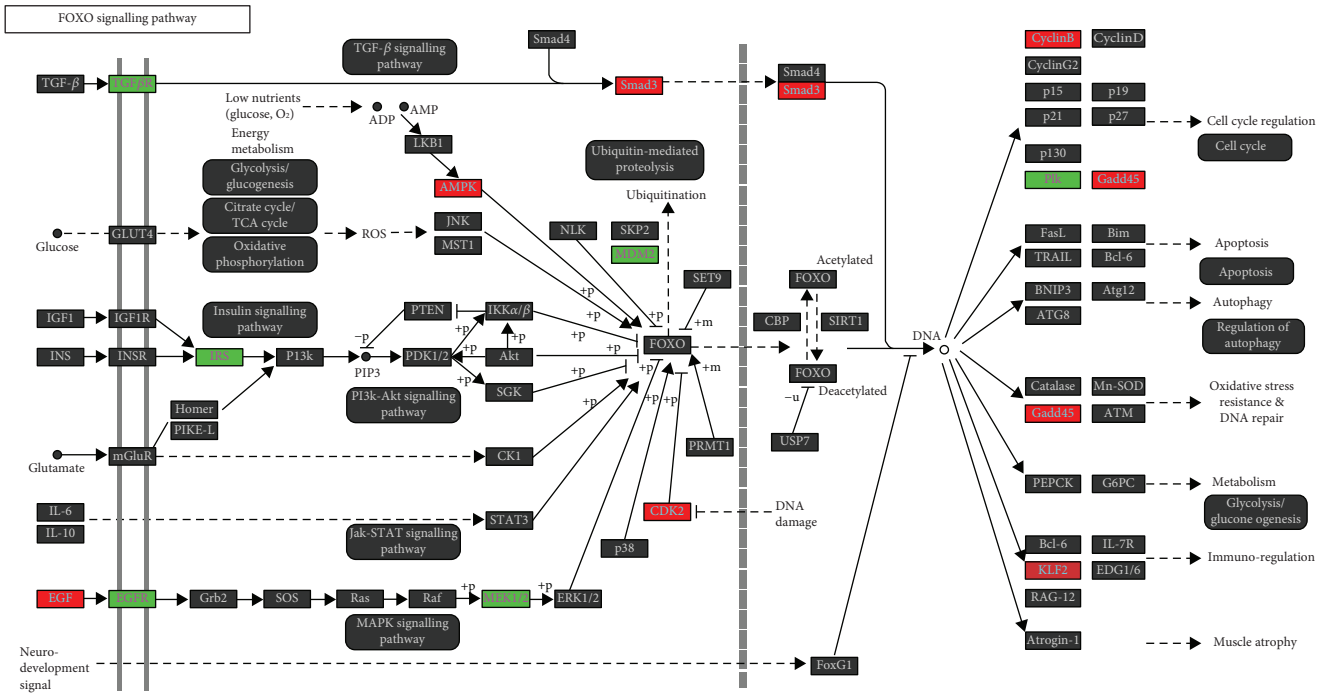
Figure 2: Continued.

(c)
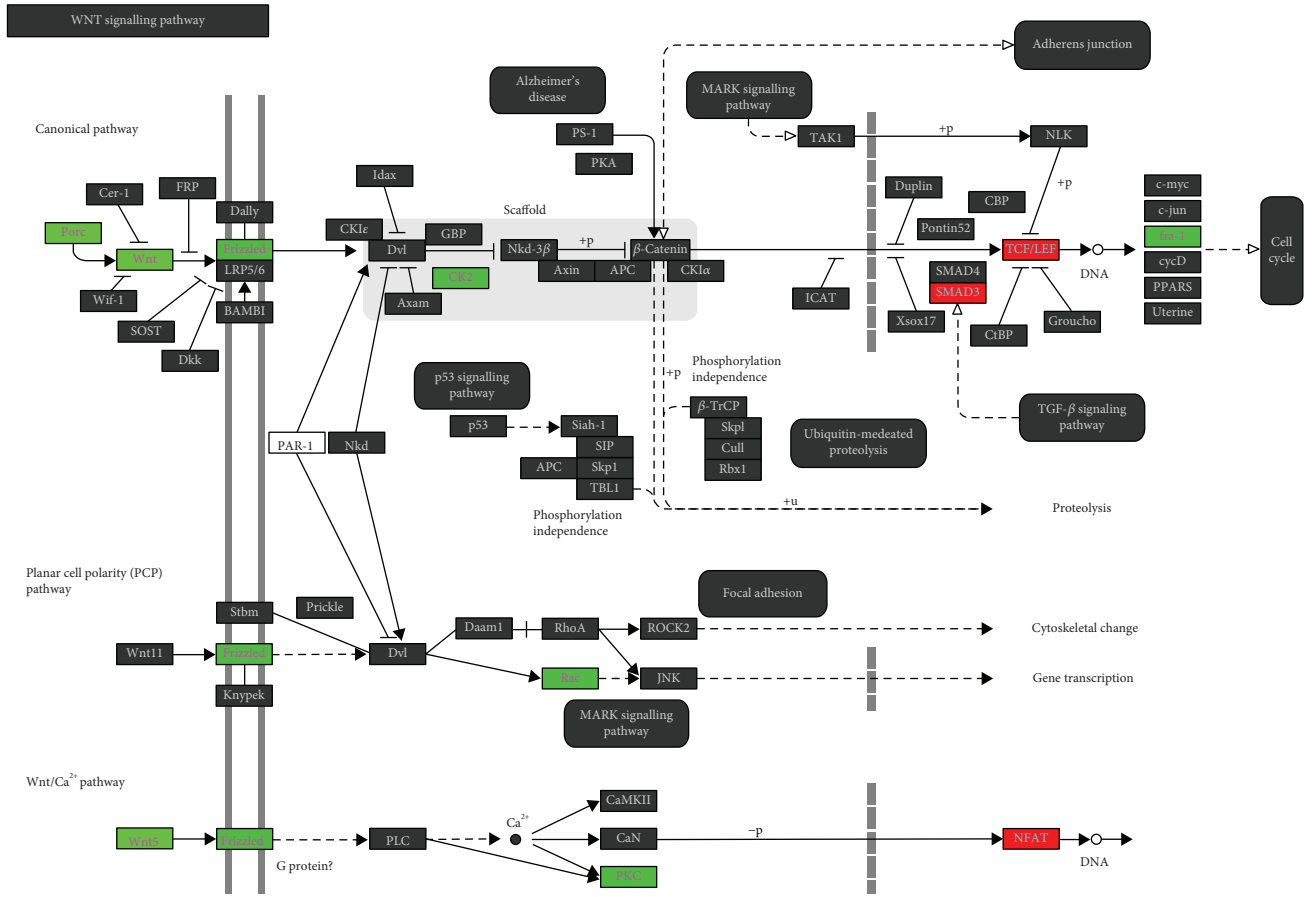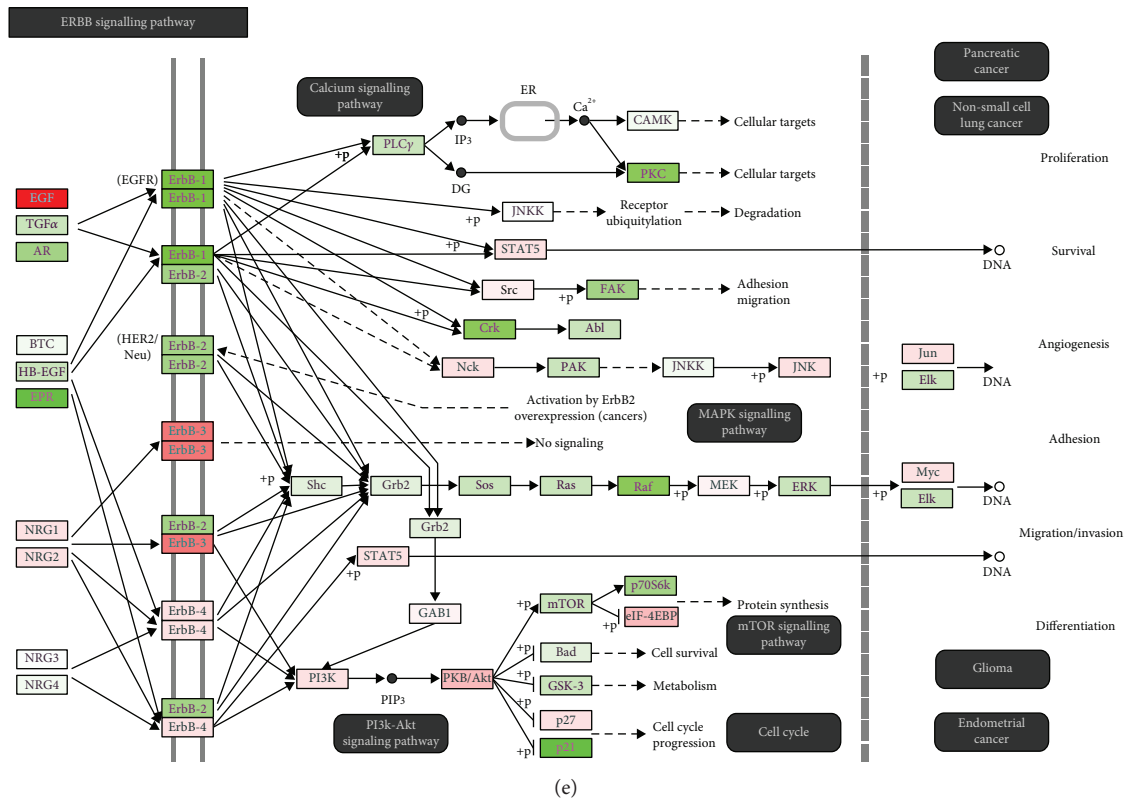

(d)

Figure 2: Continued.

(e)

FIGURE 2: Differential gene expression involved in the KEGG pathway regulated by TRF treatment, i.e., (a) cell cycle, (b) p53 signalling pathway, (c) FoxO signalling pathway, (d) Wnt signalling pathway, and (e) ErbB signalling pathway.

initiated by DNA damage and further involved in DNA repair regulated by p53 [50, 51]. Dysfunction of *RRM2B* was first reported in mitochondrial DNA depletion syndrome which caused early fatality in children [52, 53]. Kuo et al. reported that the expression of *RRM2B* was highly induced during oxidative stress in the human primer fibroblast (IMR90 cell) to stimulate antioxidant reaction [50]. Silencing of *RRM2B* expression leads to an increase in reactive oxygen species level, mitochondrial membrane depolarisation, and premature senescence in young fibroblasts. Thus, upregulation of *RRM2B* expression by TRF was suggested to stimulate the antioxidant defence in order to counteract with the increasing stress level in SIPS myoblasts.

4.2.4. Sestrins. Aside from *RRM2B*, *SESN1* was also upregulated by the TRF. Sestrins were the third recently identified hallmark of sarcopenia [54]. Sestrin expression was upregulated in DNA damaged cells, oxidative stress condition, and hypoxia [55]. *SESN1* and *SESN2* are p53-regulated genes which are involved in the regulation of autophagy and cell viability, whereas *SESN3* is a FoxO-regulated gene which in turn activated *SESN1* [56–58]. Inhibition of mTORC1 activation via the sestrin-AMPK pathway was reported to extend the lifespan of mammalia [59–61]. A more specific study was carried out to investigate the expression of sestrins towards the skeletal muscle [62]. The thorax of adult Drosophila mainly consists of skeletal muscle enriched with Drosophila sestrins (*dSesn*). *dSesn*-null flies experienced

acceleration in aging-associated degeneration, such as loss of sarcomeric structure and abnormality in mitochondria. However, treatment with vitamin E effectively prevented the deterioration in skeletal muscle and cardiac muscle by reducing the involvement of ROS [62].

GSEA and KEGG pathway analysis showed that the cell cycle was downregulated by TRF treatment in SIPS myoblasts. This finding was in contrast with the findings which reported that TRF reduced the cell cycle arrest in senescence human fibroblast cells [63]. However, according to Blagosklonny, cell cycle arrest does not absolutely refer to senescence and vice versa [64]. Cell cycle arrest actually induced the cells to proceed to another stage of the cell cycle, such as quiescence, senescence, apoptosis, motility, and differentiation [65]. Skeletal muscle satellite cells withdraw from the cell cycle for two purposes, i.e., to return to the quiescence state and carry out self-renewal or to differentiate and generate new muscle fibres. These withdrawals can be differentiated by the differential expression of genes or pathway activation. Notch signalling was activated, and *Spry1* expressions were upregulated when the cells return to the quiescence state, whereas *Delta1* expression was upregulated for the cells to differentiate after withdrawing from the cell cycle [45, 46]. However, in our study, neither *Spry1* nor *Delta1* expression was regulated by TRF treatment. Instead, the expression of *Spry4* was upregulated and the expression of *Fgf1* was downregulated by the TRF. Downregulation of *Fgf1* was once reported in the inhibition of mouse myoblast differentiation
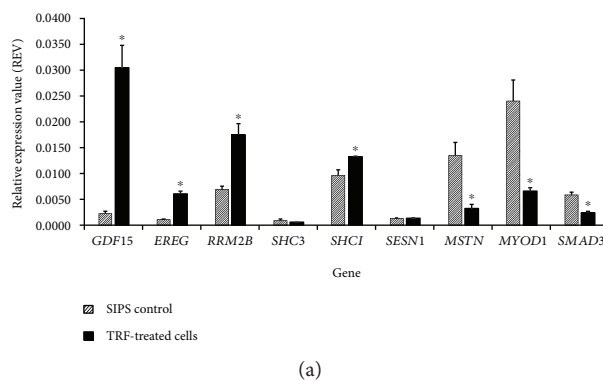
(a)

Gene	Name of Gene	Microarray		qPCR	
		Fold change	P Value	Fold change	P Value
GDF15	*Growth differentiation factor* 15	14.86	7.39E-03	14.20	4.17E-02
EREG	*Epiregulin*	5.68	2.23E-02	6.26	3.87E-02
RRM2B	*Ribonucleotide reductase M2 B (TP53 nducible)*	3.02	6.53E-03	3.82	1.27E-02
SHC3	*SHC (Src homology 2 domain containing) transforming protein* 3	2.70	3.73E-02	1.15	8.21E-01
SHC1	*SHC (Src homology 2 domain containing) transforming protein* 1	2.36	4.66E-02	1.38	3.75E-02
SESN1	*Sestrin* 1	1.87	8.10E-03	1.03	8.27E-01
MSTN	*Myostatin*	−5.46	3.24E-02	−3.16	4.27E-03
MYOD1	*Myogenic differentiation* 1	−3.08	1.28E-02	−3.30	1.75E-02
SMAD3	*SMAD family member* 3	−2.54	2.70E-03	−2.60	6.68E-03

(b)

FIGURE 3: Microarray result validation. (a) The REV value and (b) the fold change were consistent between the microarray analysis and the qPCR analysis. The REV data are shown as mean ± SEM. $^*p < 0.05$ compared to SIPS control.

[66, 67]. Furthermore, upregulation of *Fgf2* was reported in the activation of satellite cells and in turn increased the expression of *Twist2* and *Spry4*, thus inhibiting the differentiation of mouse mesenchyme stem cells [68, 69]. Hence, we proposed that the withdrawal from the cell cycle of SIPS myoblasts posttreated with TRF was not due to differentiation, as differentiation was inhibited by downregulation of *Fgf1* and upregulation of *Spry4*. Instead, the TRF promoted the myoblast cells to exit the cell cycle and return to their quiescence state.

4.2.5. Myogenic Regulatory Factor (MRF) and Wnt Signalling Pathway. The effect of TRF towards expression of the myogenic regulatory factor (MRF) was in line with the suggestion that the myoblasts exit the cell cycle for the maintenance of the quiescence cell population. The myogenic potential of satellite cells will highly depend on the expression of *Pax* and MRF (*MyoD*, *Myf5*, *myogenin*, and *MRF4*) [70]. Expression of *Myf5* and/or *MyoD* was upregulated during the activation of satellite cells to the myoblast and early myogenic differentiation [71–73], whereas differentiation of myoblasts would upregulate the expression of *myogenin* and *MRF4* (also known as *Myf6*) [73]. TRF treatment has downregulated

the expression of *Myf5*, *MyoD1*, and *Myf6*, which means that TRF treatment did not promote the myoblast cells to withdraw from the cell cycle for differentiation.

The expression of MRF was regulated by the Wnt signalling pathway during the development of the embryo. However, in the adult skeletal muscle, the canonical Wnt signalling pathway regulated the differentiation of muscle satellite cells, whereas the noncanonical Wnt signalling pathway mediated the self-renewal of satellite stem cells and the growth of muscle fibres [74]. Our GSEA analysis revealed that the noncanonical Wnt signalling pathway was significantly regulated by TRF treatment. Among the Wnt involved in the noncanonical Wnt signalling pathway (*Wnt4*, *Wnt5a*, *Wnt5b*, *Wnt8a*, *Wnt8b*, *Wnt10a*, and *Wnt10b*), TRF treatment has upregulated the expression of *Wnt5a* and *Wnt7b*. Studies reported that Wnt were expressed at different times of injury in order to promote regeneration [75, 76]. *Wnt5a*, *Wnt5b*, and *Wnt7a* were upregulated at the early stage of regeneration, and *Wnt7b* and *Wnt3a* were regulated at the final stage after injury. The regulation of the Wnt signalling pathway, cell cycle, and p53 signalling by TRF treatment suggested the promotion of satellite cells to return to the quiescence state and maintain the population of quiescence cells.

4.2.6. Growth Differentiation Factor 15 (GDF15). Another critical finding of our microarray analysis was the expression of GDF15 which was highly upregulated by 14.9-fold. The expression of *GDF15* and its mechanism involved in the CHQ5B cells were not well studied. Previously, *GDF15* was reported as a negative regulator in skeletal muscle growth [77, 78]. Senescence, smoking, and environmental factors would increase the GDF15 level [79]. The levels of GDF15 increased in patients experiencing intensive care unit acquired muscle weakness (ICUAW) or muscle atrophy [80, 81]. However, aside from a high level of GDF15, SMAD2/3 was activated by GDF15 in the ICUAW patients and this was in contrast with our findings. Findings showed that SMAD3 expression was downregulated by TRF treatment. GDF15 prevents the ROS production and exhibits an antia-poptotic effect and cell proliferation regulation [78, 82, 83]. Hence, the conclusion on the activity of GDF15 towards CHQ5B cells remains unknown due to its lack of investigation. Development of therapeutic interventions with GDF15 or anti-GDF15 agents remains difficult until the mechanism that drives its activity is revealed with more evidences.

5. Conclusions

At present, most of the studies focused on the effect of tocopherol on the skeletal muscles, but less *in vitro* findings were reported on the effects of tocotrienol towards human skeletal muscle cells and the mechanism involved remains unclear. Recent studies showed that muscle regeneration does not solely depend on the myogenic proliferation of the satellite cell as prolonged imbalance between the expansion and maintenance of the satellite stem cell population leads to impaired muscle regeneration [46]. Our findings proposed that TRF treatment not only promotes the proliferation capacity of SIPS myoblasts through regulation of the ErbB signalling pathway (upregulation of expression of *EREG*, *SHC1*, and *SHC3*) and FoxO signalling pathway (downregu-lation of expression of *MSTN* and *SMAD3*). At the same time, TRF treatment is proposed to modulate the renewal of satellite cells through regulation of p53 signalling (upregulation of *RRM2B* and *SESN1*), cell cycle, Wnt signalling path-way, and expression of MRF. Even though these findings were exciting, more extensive studies such as proteomic anal-ysis and various time points of TRF treatments in the SIPS myoblast model are suggested in order to give a more thor-ough idea on the involvement of TRF in the regenerative capacity and mechanism of skeletal muscles.

Abbreviations

AGCC: Affymetrix GeneChip Command Console
aRNA: Amplified RNA
CEL: Cell intensity file
Ct: Threshold cycle
DMEM: Dulbecco's modified Eagle medium
EGFR: Epidermal growth factor receptor
EREG: Epiregulin
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
GDF15: Growth differentiation factor 15
GSEA: Gene set enrichment analysis
H_2O_2: Hydrogen peroxide
KEGG: Kyoto Encyclopedia of Genes and Genomes
MRF: Myogenic regulatory factor
Myf5:   Myogenic factor 5
MyoD: Myogenic differentiation 1
PCA: Principal component analysis
qPCR: Quantitative real-time polymerase chain reaction
REV: Relative expression value
RIN: RNA integrity number
SIPS: Stress-induced premature senescence
SMOX: Spermine oxidase
TRF: Tocotrienol-rich fraction.

Data Availability

The data used to support the findings of this study are included within the article and supplementary information files.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Authors' Contributions

JJL, NAK, and WZWN conceived and designed the experi-ments. VM provided the human satellite cells CHQ5B and technical guidance on CHQ5B cell culture. JJL performed the experiments and analysed the data. JJL and NAK drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgments

Supplementary Materials

Figure S01: RNA integrity number (RIN) assessed by the electropherogram bioanalyzer for the (a) untreated young control cells, (b) SIPS control, and (c) TRF-posttreated SIPS cells. Figure S02: the specificity of primers assessed by (a–j) standard curve and melt curve analysis of each pair of primers and (k) agarose gel electrophoresis that was performed on the PCR products. Table S01: the com-plete list of 41 differentially expressed genes associated with stress-induced premature senescent control cells as compared to untreated young control cells (fold change < -1.5 or fold change > 1.5; $p < 0.05$). Table S02: the complete list of 905 differentially expressed genes associated with TRF-posttreated SIPS cells as compared to SIPS control cells (fold change < -1.5 or fold change > 1.5; $p < 0.05$). *(Supplementary Materials)*

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