Current Aging Science, 2018, *11*, 165-172

RESEARCH ARTICLE

Altered Gene Expression of Muscle Satellite Cells Contributes to Age-related Sarcopenia in Mice

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ARTICLE HISTORY

Received: March 19, 2018
Revised: June 21, 2018
Accepted: June 21, 2018

DOI:
10.2174/1874609811666180925104241

Abstract: ***Background*:** During aging, muscle tissue undergoes profound changes which lead to a decline in its functional and regenerative capacity. We utilized global gene expression analysis and gene set enrichment analysis to characterize gene expression changes in aging muscle satellite cells.

***Method*:** Gene expression data; obtained from Affymetrix Mouse Genome 430 2.0 Array, for 14 mouse muscle satellite cell samples (5 young, 4 middle-aged, and 5 aged), were retrieved from public Gene Expression Omnibus repository. List of differentially expressed genes was generated based on 0.05 multiple-testing-adjusted p-value and 2-fold FC cut-off values. Functional profiling of genes was carried out using PANTHER Classification System.

***Results*:** We have found several differentially expressed genes in satellite cells derived from aged mice compared to young ones. The gene expression changes increased progressively with time, and the majority of the differentially expressed genes were upregulated during aging. While the down-regulated genes could not be correlated with specific biological processes the upregulated ones could be associated with muscle differentiation-, inflammation- or fibrosis-related processes. The latter two processes encompass the senescence-associated secretory phenotype for satellite cells which alters the tissue microenvironment and contributes to inflammation and fibrosis observed in aging muscle.

***Conclusion*:** Our analysis reveals that by altering gene expression pattern and expressing inflammatory mediators and extracellular matrix components, these cells can directly contribute to muscle wasting in aged mice.

Keywords: Satellite cell, aging, senescence-associated secretory phenotype, fibrosis, muscle, inflammation.

1. INTRODUCTION

Muscle mononuclear stem cells or Satellite Cells (SCs) are the major stem cells in postnatal skeletal muscle [1]. They play a role in the maintenance of muscle mass, remodeling, and hypertrophy during the lifespan. SCs are located in the space between the mature myofiber plasma membrane and basement membranes and are characterized by Pax7 positivity, MyoD1 negativity, and $CD45^{-}Sca\text{-}1^{-}CD11b^{-}CXCR4^{+}\beta1\text{-}integrin^{+}$ cell surface marker expression in rodents [2, 3]. SCs serve as a nuclear reserve and are critical for muscle regeneration following an injury, and exercise-induced muscle hypertrophy. In normal conditions, SCs are in a quiescent state, which is a state of cellular hibernation. Upon stimuli; such as myofiber damage, mechanical stretching, cytokines, electrical stimulation, nitric oxide or growth factors (i.e. fibroblast growth factor (FGF), Hepatocyte Growth Factor (HGF) and insulin-like growth factor (IGF)-1), SCs are temporarily activated, and become $MyoD1^{-/+}$ [4]. Activated SCs enter the cell cycle and undergo self-renewing proliferation, giving rise to additional SCs and further differentiating $MyoD1^{+}$myoblasts [2].

Aging is accompanied by a time-dependent accumulation of damage in the cells. Long-lived stem cell populations can evade the effects of aging by quiescence (state of temporal cell cycle arrest), which provides protection against the stress associated with cellular proliferation, thereby, limiting cellular damage [5]. However, as described earlier, these cells are not dormant and can start proliferation upon stimulation. Aging is associated with the inability to maintain stem cell quiescence, which increases the chances of stem cell damage, and results in a loss of stem cell self-renewal and regenerative capacity [6]. These senescent cells are metabolically active, but irreversibly lose their proliferative capacity, and undergo extensive changes in protein expression and secretion; such as inflammatory cytokines, growth factors, Extracellular Matrix (ECM) components and proteases, ultimately developing the Senescence-Associated Secretory Phenotype (SASP) [7]. During aging, senescent cells accu-

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mulate, and the secreted pro-inflammatory cytokines, *e.g*. Tumor Necrosis Factor Alpha (TNFα) and Interleukin 6 (IL6), increase tissue inflammation [8]. Prolonged inflammation inflicts damage to tissues, which together with increased stem senescence and decreased regeneration, leads to tissue degeneration.

In skeletal muscle, aging is manifested by loss of SCs [9]. Moreover, the remaining SCs present reduced capacity for self-renewal, and stretch-induced activation leading to sarcopenia [10, 11]. These age-related degenerative losses of muscle mass, quality, and function take place in the absence of any underlying disease. Skeletal muscle is eventually replaced by fatty and fibrous tissue, which results in functional impairment of the muscle and hence, physical disability. Aging is also accompanied by constant, low-level inflammation in the body of the elderly [12, 13]. While temporal inflammation is required for SC activation and proliferation during muscle regeneration, continuous inflammation in SC niche depletes SCs, triggers protein catabolism, and impairs anabolic processes of skeletal muscle [14, 15]. Thus, increased inflammation and impaired SC function are thought to result in the observed sarcopenia of the elderly [16, 17].

In the present paper, using global gene expression and gene set enrichment analysis, we aimed to characterize the aging process in SCs. We found that aging is associated with increased expression of genes involved in myogenesis and muscle contraction, indicating loss of the stem cell properties. Moreover, we present the unique SASP for SCs, characterized by increased expression of various growth factors, ECM-, and inflammation-related genes.

2. MATERIALS AND METHODS

2.1. Data Processing

Gene expression data, obtained from Affymetrix Mouse Genome 430 2.0 Array, for 14 mouse muscle satellite cell (isolated by FACS based on $CD45^{-}Sca\text{-}1^{-}Ter119^{-}$ $Cxcr4^{+}$ $\beta1\text{-}integrin^{+}$ cell surface marker pattern) samples (5 young, 2-month-old; 4 middle-aged, 12-month-old and 5 aged, 24-month-old samples) were retrieved from public Gene Expression Omnibus repository (GEO series GSE50821). For detailed cell isolation and hybridization protocol, see Sinha M supplementary materials [18]. Gene expression data from middle-aged and aged satellite cells were compared to young samples using the GEO2R platform (https://www.ncbi.nlm.nih.gov/geo/geo2r/). Equal value intensity distribution and cross-comparability of individual microarrays were confirmed using box-and-whisker plots. The significance of fold changes (FC) was tested with Moderated t-test adjusted by Benjamini-Hochberg False Discovery Rate (FDR) correction for multiple testing. Data were imported in Microsoft Access and list of differentially expressed genes (DEG) was generated by removing non-significantly changed transcripts based on 0.05 multiple-testing-adjusted p-value and 2-fold FC cut-off value.

2.2. Functional Profiling

In order to gain insight into the biological function of the given transcripts, we used the PANTHER (Protein Analysis Through Evolutionary Relationships) Classification System (http://pantherdb.org/) to identify over-represented canonical pathways (Reactome pathways), biological processes and functions (Gene Ontology) among the DEGs. Duplicated transcripts were removed from the DEG list, and statistically significant enrichment of genes within Reactome pathways and GO categories was determined using Fisher's Exact test with FDR multiple test correction (FDR< 0.05).

2.3. Clustering and Visualization of Data

DEGs between young, middle-aged and aged SCs were clustered with Cluster software (M. Eisen, 1999 http://rana.lbl.gov/EisenSoftware.htm) using a Self-Organizing Map (SOM) with 1,000,000 iterations and 4 nodes. The log2 transformed FC values of genes in selected PANTHER analysis terms and the result of SOM clustering were visualized using heatmaps generated by TreeView software (M. Eisen, 1999 (http://rana.lbl.gov/EisenSoftware.htm).

3. RESULTS

3.1. Differentially Expressed Genes During SC Aging

Based on 2-fold change and $p < 0.05$ significance level in all age groups, we identified 606 transcripts with altered gene expression during mouse SCs aging in the analyzed GEO database. 136 transcripts showed decreased gene expression in aged samples and 137 in middle-aged group. The top 50 upregulated and downregulated genes in aged SCs are listed in supplementary Table **1** and **2**, respectively. The

Table 1. PANTHER GO-Slim Cellular Component overrepresentation test of genes upregulated in aged SCs compared to young ones. REF#: number of reference genes in the given category; F#: number of genes found among the upregulated genes in aging SCs; FDR: False Discovery Rate value calculated by Benjamini-Hochberg procedure for multiple test correction (FDR < 0.05).

PANTHER GO-Slim Cellular Component	REF#	F#	Expected	Fold Enrichment	FDR
Lysosome	66	5	1	5	4.43E-02
Extracellular matrix	95	7	1.4	4.9	1.09E-02
Actin cytoskeleton	180	11	2.7	4.0	3.16E-03
Extracellular space	643	29	9.8	3	1.17E-05
Extracellular region	780	40	11.8	3.4	3.40E-09

Table 2. PANTHER GO-Slim Biological Process overrepresentation test of genes upregulated in aged SCs compared to young ones. REF#: number of reference genes in the given category; F#: number of genes found among the upregulated genes in aging SCs; FDR: False Discovery Rate value calculated by Benjamini-Hochberg procedure for multiple test correction (FDR < 0.05).

PANTHER GO-Slim Biological Process	REF#	F#	Expected	Fold Enrichment	FDR
Response to interferon-gamma	60	8	0.91	8.78	4.76E-04
Immune system process	278	11	4.22	2.61	4.64E-02
Muscle contraction	107	13	1.62	8.00	7.56E-06
Cell-matrix adhesion	51	6	0.77	7.75	5.54E-03
Cell growth	45	5	0.68	7.32	1.66E-02
Growth	51	5	0.77	6.46	2.13E-02
Cytokine-mediated signaling pathway	69	6	1.05	5.73	1.58E-02
Response to external stimulus	319	19	4.84	3.92	1.14E-04
Locomotion	256	14	3.89	3.60	1.87E-03
Cellular component morphogenesis	331	18	5.03	3.58	4.88E-04
Anatomical structuremorphogenesis	461	21	7.00	3.00	6.69E-04
Developmental process	1580	43	23.99	1.79	5.83E-03
Nervous system development	231	10	3.51	2.85	3.93E-02
MAPK cascade	324	14	4.92	2.85	1.25E-02
Proteolysis	427	16	6.48	2.47	1.82E-02
Cellular component movement	414	15	6.29	2.39	2.79E-02
Cell differentiation	458	16	6.95	2.30	3.44E-02

average FC of decreased transcripts was -1.97 ± 1.31 in aged and -2.18 ± 1.25 in middle-aged group. The median FC value of decreased transcripts was -2.03 in aged and -2.15 in middle-aged groups. 470 transcripts showed increased gene expression in aged samples and 469 in middle-aged group. The average FC of increased transcripts was 3.27 ± 1.84 in aged and 2.82 ± 1.74 in middle-aged group. The median FC value of increased transcripts was 2.83 in aged and 2.45 in middle-aged group. Taken together, our results indicate progressive increasing changes in gene expression during SC aging.

3.2. Functional Profiling of Differentially Expressed Genes

To gain an insight into the biological processes that might be affected by aging in SCs, we carried out gene set enrichment analysis using the PANTHER Classification System tool. Our analysis revealed no statistically overrepresented GO-Slim Biological Process, GO-Slim Cellular Component or Reactome pathway among the genes that were downregulated in the middle-aged and aged SCs, as compared to SCs isolated from young mice. The same analysis revealed 5 GO-Slim Cellular Component (Table **1**), *e.g.*, "actin cytoskeleton" and "extracellular space", 17 GO-Slim Biological Process categories (Table **2**); such as "Response to interferon-gamma" and "Muscle contraction" among the genes that were upregulated in the middle-aged and aged SCs when compared to young cells. We also found 22 Reactome pathways (*e.g.*, "ECM organization", the non-protein ECM component "Keratan sulfate metabolism", and "Elastic fibre formation") which were significantly overrepresented among the upregulated genes (Table **3**). By investigating the genes belonging to Reactome pathways and PANTHER GO-Slim categories, we identified 18 genes associated with "Muscle contraction". 10 of them (Myl1, Mylpf, Tnni2, Tnnt3, Tnnc2, Tpm1, Tpm2, Myh4, Myh, and Myo1d) were already reported as upregulated genes in aged, 24-month-old SCs by Sinha M and coworkers, and therefore, not shown in Fig. (**1**) [18]. Genes related to muscle contraction and myogenic differentiation showed a significantly increased expression during SC aging (FC middle-aged 2 *vs*. FC aged 4, $p < 6.8e^{-6}$). We identified 22 genes related to "ECM organization" (*e.g.*, Adamts2, Col8a1, Icam1, Itgb5, and Thbs1) which were significantly upregulated during SC aging (Fig. **2**). Based on our analysis, functional profiling of the DEGs reveals increased extracellular protein secretion, myogenic differentiation, ECM protein production, deposition, and maturation in aging SC cells.

3.3. Identification of SASPs in SCs

Since increased protein secretion and ECM-related protein expression is a feature of SASP, we extended the search in our DEG list for genes which are known SASP members

Table 3. PANTHER Reactome pathways overrepresentation test of genes upregulated in aged SCs compared to young ones. REF#: number of reference genes in the given category; F#: number of genes found among the upregulated genes in aging SCs; False Discovery Rate value calculated by Benjamini-Hochberg procedure for multiple test correction (FDR < 0.05).

Reactome Pathways	REF#	F#	Expected	Fold Enrichment	FDR
Keratan sulfate degradation	11	6	0.2	35.9	5.82E-05
Keratan sulfate/keratin metabolism	30	8	0.5	17.6	5.77E-05
Glycosaminoglycan metabolism	112	11	1.7	6.5	5.34E-04
Metabolism of carbohydrates	259	18	3.9	4.6	8.32E-05
Metabolism	1714	51	26.0	2.0	9.87E-04
Creatine metabolism	7	3	0.1	28.2	2.65E-02
Cross-presentation of particulate exogenous antigens (phagosomes)	8	3	0.1	24.7	3.44E-02
Purine catabolism	11	4	0.2	24.0	6.85E-03
Purine metabolism	35	5	0.5	9.4	2.35E-02
Keratan sulfate biosynthesis	25	7	0.4	18.4	9.68E-05
Smooth Muscle Contraction	30	6	0.5	13.2	2.40E-03
Muscle contraction	156	11	2.4	4.6	5.69E-03
Striated Muscle Contraction	33	6	0.5	12.0	3.48E-03
Molecules associated with elastic fibres	32	5	0.5	10.3	1.80E-02
Elastic fibre formation	35	6	0.5	11.3	4.26E-03
Extracellular matrix organization	260	22	4.0	5.6	4.92E-07
Chemokine receptors bind chemokines	50	6	0.8	7.9	1.75E-02
Peptide ligand-binding receptors	179	11	2.7	4.1	1.45E-02
Collagen biosynthesis and modifying enzymes	62	6	0.9	6.4	3.53E-02
Collagen formation	89	8	1.4	5.9	1.13E-02
Integrin cell surface interactions	76	7	1.2	6.1	1.96E-02
Degradation of the extracellular matrix	126	9	1.9	4.7	1.80E-02

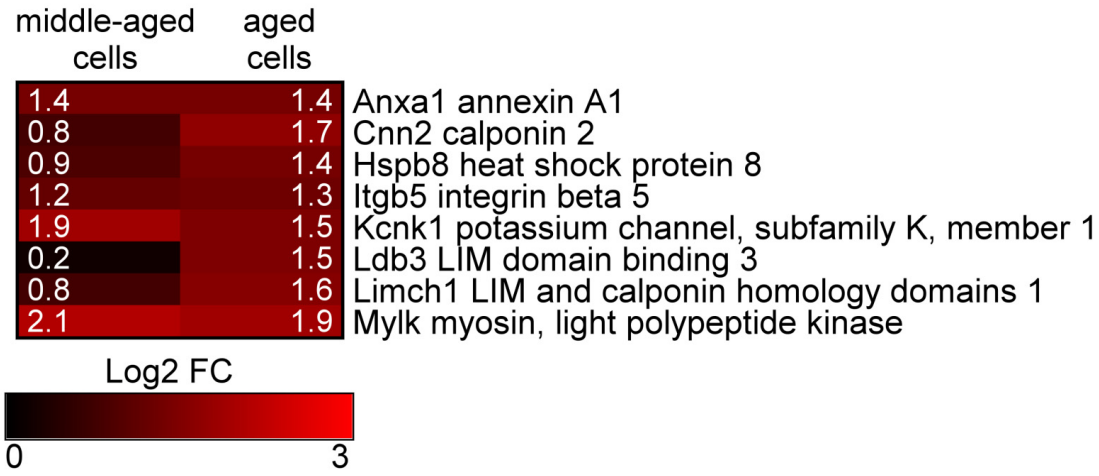

Fig. (1). Heatmap of 8 muscle contraction-related genes. Based on combined Reactome pathways and PANTHER GO-Slim category 8 new upregulated genes were identified in aged SCs associated with muscle contraction. Heatmap displays the log2 transformed FC values of middle-aged and aged mouse samples compared to young cells. Numbers inside the heatmap indicate the log2 transformed FC values of the given transcripts. FC: Fold Change.

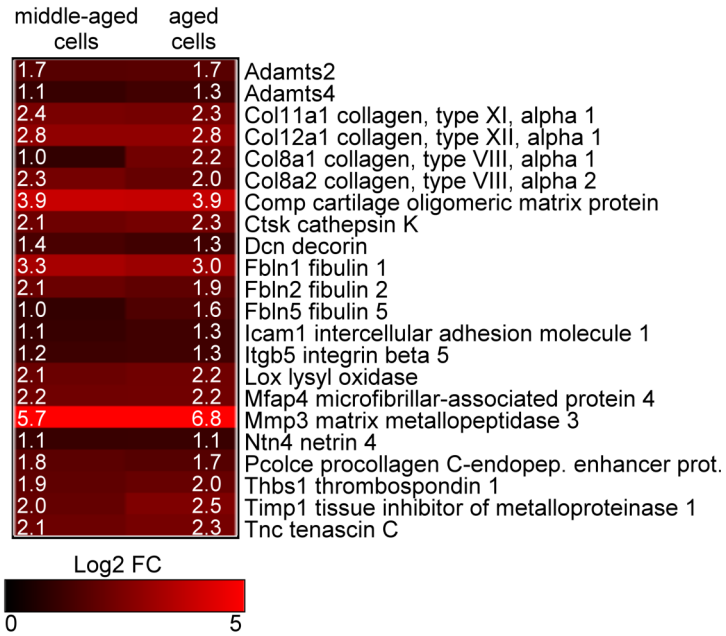

Fig. (2). Heatmap of 22 ECM organization related genes. Based on Reactome pathways 22 upregulated genes were identified in aged SCs associated with ECM organization. Heatmap displays the log2 transformed FC values of middle-aged and aged mouse samples compared to young cells. Numbers inside the heatmap indicate the log2 transformed FC values of the given transcripts. FC: Fold Change.

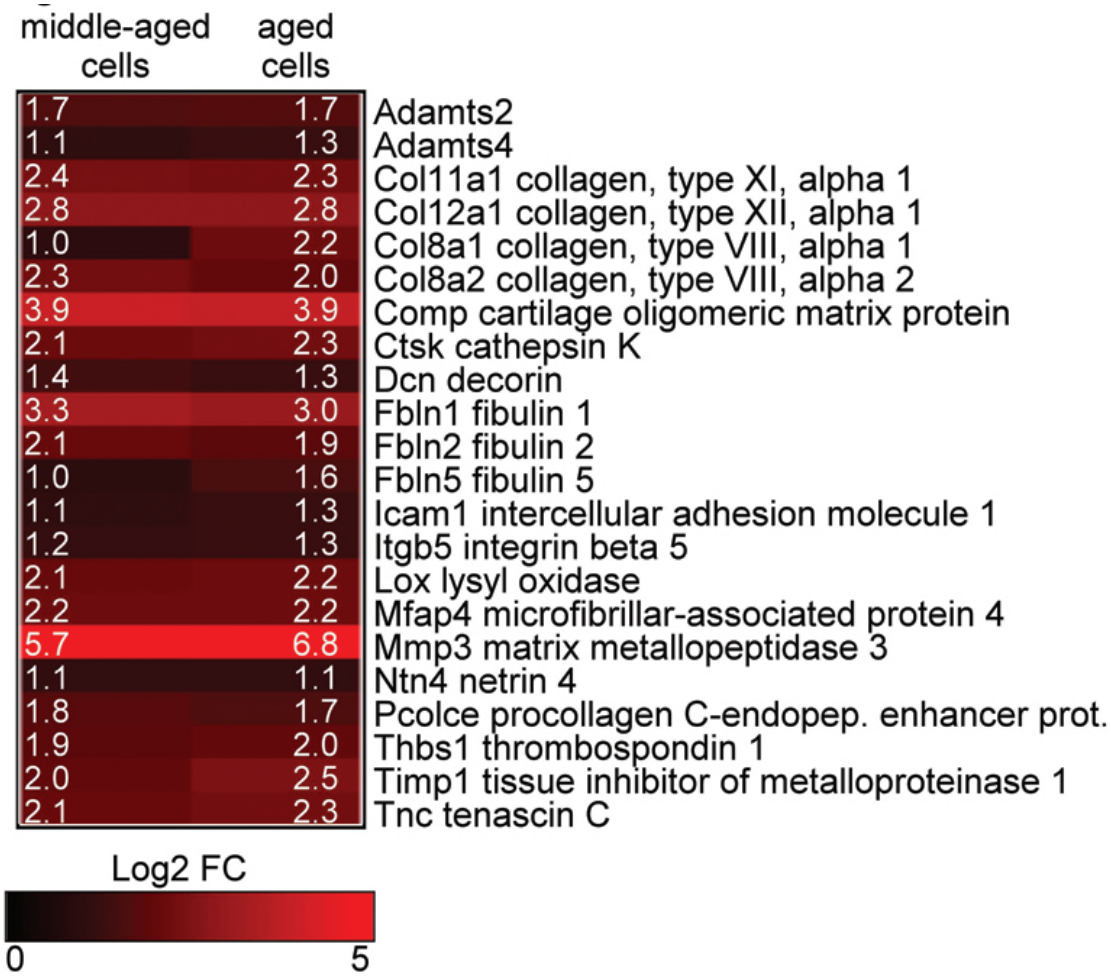

Fig. (3). Heatmap of 35 SASP genes. Heatmap displays the log2 transformed FC values of middle-aged and aged mouse samples compared to young cells. Numbers inside the heatmap indicate the log2 transformed FC values of the given transcripts. The 35 genes can be divided into six categories and two sub-categories as indicated left to the panel. FC: Fold Change.

in epithelial cells and fibroblasts [7]. The SASP expression profile may differ in various cell types, therefore, we searched for reported SASP member-related genes as well. Besides the already in Fig. (**2**) presented ECM-associated genes we found 35 genes which were reported as SASP members in other cell types, or are related to an already categorized SASP gene. The expression pattern and log2 transformed fold-change values of the individual transcript are shown in Fig. (**3**). The found genes can be divided into 6 main categories: interleukins, chemokines, growth factors, proteases and regulators, cell-cell interaction, and ECM components and processing enzymes.

4. DISCUSSION

In our study, we describe how mouse-derived SCs undergo profound gene expression changes during aging. These alterations in gene expression profile result in increased myogenic differentiation, ECM deposition, and maturation. In previous experiments, transcriptional analysis of sorted SCs isolated from old (22-, 24-month-old) mice, revealed that expression of myoD1; a marker of cycling and differentiation, was higher, while quiescent markers (cyclin-dependent kinase inhibitor 1B (cdkn1b/p27Kip1) and sprouty 1 (Spry1); an inhibitor of fibroblast growth factor (FGF) signaling) were expressed at lower level, compared to cells isolated from 6-month-old adults [19]. These gene expression changes were assumed to impair the ability of aged SCs to retain the quiescent state, driving them toward continuous proliferation and differentiation, leading to the depletion of the SC pool. In another study, transplantation of FACS-sorted SCs from geriatric mice (28-, 32-month-old) into young recipients showed that those SCs were unable to activate upon stimulation, enforcing the significance of cell-intrinsic alterations during SC aging [6]. One of the factors that the authors found to contribute to the reduced regenerative capacity was the upregulation of p16INK4A cyclin-dependent kinase inhibitor. In our analyis, the expression of myoD1, cdkn1b, Pax7, Spry1, and p16INK4A were not altered in the investigated 12- and 24-month-old mice groups (data not shown). However, statistical overrepresentation tests showed the upregulation of 18 genes associated with the actin-myosin machinery and muscle contraction. This indicates ongoing myogenic differentiation in SCs from old mice. Our results are also partially overlapping with the findings of Sinha and coworkers, who have identified 15 myocyte differentiation marker genes; Mylpf, Myl1, Mylc2b, TnnC2, and Tpm1-2, among others in aged SCs [18].

Senescent cells secrete a set of molecules which are defined as the SASP profile of the given cell type. These molecules encompass; among others, inflammatory cytokines, chemokines, growth factors, ECM components and modifying enzymes, which alter the microenvironment of aging cells. Our analysis confirmed the increased expression of IL6, IL1 superfamily member IL33, and Ccl2, found by Oh and coworkers. In addition, it revealed increased chemokine (*e.g.*, Cx3cl1, Ccl11-19, and Cxcl9-16) expression during SC aging [20]. These molecules; activating broad spectrum of leukocytes and attracting neutrophils and macrophages, are contributing to the development of inflammation. The ECM modifier lysyl oxidase (LOX) enzyme is 4-fold upregulated in middle-aged and aged SCs [21]. Interestingly, LOX was shown to possess chemotactic activity for human peripheral blood mononuclear cells; accordingly, an increase in extracellular LOX protein amount might recruit additional inflammatory cells to aging muscle [22]. Another ECM modifier molecule which was two-fold upregulated in aging SCs was the secreted cysteine protease cathepsin K (Ctsk). Ctsk is one of the most potent mammalian collagenases, and its expression is induced by inflammatory cytokines [23]. Using Ctsk knockout mice, Ogasawara and coworkers found a decreased inflammatory marker expression, leukocyte migration and fibrosis after cardiotoxin-induced muscle injury in the absence of Ctsk, indicating a positive role of Ctsk in these processes [24]. Therefore, increased Ctsk expression in aging SCs could aggravate muscle inflammation in old mice. Although coordinated inflammation is required for muscle regeneration upon injury, the signs of persistent inflammation and absence of anti-inflammatory markers indicate aberrant muscle regeneration in aged muscles. Moreover, we have found increased expression of the protein cross-linking, ECM modifier enzyme tissue transglutaminase 2 (TG2) in aged SCs compared to young ones (3 and 3.4 fold upregulation in middle-aged and aged cells, respectively). TG2 expression is normally undetectable in postnatal skeletal muscle, but was found to be expressed in idiopathic inflammatory myopathies [25]. TG2 is known to be induced by several inflammatory cytokines, it also contributes to the initiation of inflammation by activating NF-κB pathway via posttranslational cross-linking and inactivation of NF-κB inhibitor (NF-κBi),which may contribute to the elevated NF-κB activity observed in aged SCs [26, 20]. Interestingly, we also found upregulation of NF-κBiε in aging SCs (2.5 and 2.6 fold increase in middle-aged and aged cells, respectively) which could partially limit harmful muscle inflammation. Inflammation induces ECM production in activated fibroblasts, which normally take place during muscle regeneration, however, this can lead to muscle fibrosis when the inflammation does not resolve properly [27]. In several pathological conditions, TGF-β was shown to augment epithelial-to-mesenchymal transition, thereby increasing the number of myofibroblast, resulting in the deposition of collagens, fibronectin, and proteoglycans, leading to fibrosis [28, 29].Through posttranslational modifications, TG2 plays a role in latent TGF-β production and activation; therefore, increased TG2 expression may promote muscle fibrosis [30, 31]. Not only fibroblasts are capable of ECM production, SCs were also shown to deposit collagen [32]. In our analysis, we detected upregulation of several of the ECM members; such as collagen 8, 11 and 12, matrix Gla protein, thrombospondin 2-4 and the Small Leucine-Rich Repeat Proteins (SLRPs), fibromodulin, osteomodulin, osteoglycin, and asporin in aged SCs. Additionally, exposure of SCs to an aged myomatrix directly inhibits myogenicity, and promotes a fibrogenic conversion of SCs, establishing a vicious circle of fibrotic transformation [33]. TG2 was implicated in ECM aging and subsequent ECM function decline by establishing protease resistant ε-(γ-glutamyl)-lysine crosslinks between several ECM components suggesting possible role in muscle aging [34]. Inhibition of TG2 activity proved to be efficient in the treatment of experimental fibrotic lung and kidney disease models in animals, and enzymatic digestion of ECM proteins is an emerging technique to alleviate symptoms of Dupuytren contracture, a disease characterized by fibrotic

scar tissue accumulation and increased ECM stiffness [35-37].Therefore, inhibition of TG2 activity or removal of isopeptide crosslinks might offer a new therapeutic approach to slow down muscle fibrosis at old age.

All in all, our analysis of aging SCs revealed increased expression of genes involved in myogenic differentiation, accompanied by an elevated inflammatory cytokine-chemokine production, in addition to increased ECM protein and ECM modifying enzyme expression, which are hallmarks of muscle fibrosis. Together, these changes can contribute to the decrease in function and regenerative capacity of muscle fibers observed in sarcopenia at old age.

Regarding the limitation of the study, the microarray technology detects RNA transcripts, and has a decreased sensitivity in detecting low-abundance genes; therefore, it needs to be later verified by more sensitive quantitative PCR and protein detecting methods; such as Western blot and ELISA. In spite of these limitations, our study reveals novel features of satellite cell aging.

CONCLUSION

In the present study, we describe the gene expression changes taking place in satellite cells during aging. We have found that aging leads to profound alterations, including increased expression of myogenic differentiation-, inflammation-, and fibrosis-related genes. The latter two categories represent the senescence-associated secretory phenotype for satellite cells. The altered gene expression pattern leads to decreased stem cell function and muscle regeneration capacity which results in age-associated muscle wasting.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

This work was supported by the National Research, Development and Innovation Office (124244), by the GINOP-2.3.2-15-2016-00006 project and the European Union project titled: Institutional Developments for Intelligent Specialization program (grant number: EFOP-3.6.1-16-2016-00022 “Debrecen Venture Catapult Program”). Zsolt Sarang was a recipient of Lajos Szodoray fellowship given by the University of Debrecen.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher’s web site along with the published article.

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