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Original article

Restoration of miR-223-3p expression in aged mouse uteri with Samul-tang administration

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ABSTRACT

Background: During the secretory phase of the estrous cycle, endometrium senescence is accompanied by biological mechanisms such as metabolic dysfunction and epigenetic changes, leading to decreased embryo receptivity and implantation failure. Samul-tang has been reported to improve implantation potential in aged mice.

Methods: To uncover the age-related changes in the transcriptomes, we performed QuantSeq 3′mRNA sequencing to compare the mRNA expression patterns in the uteri between young and old mice. Young and old female BALB/c mice were administered distilled water or Samul-tang for 4 weeks, and the corresponding effects on the uteri of aged mice were investigated.

Results: We found 586 differentially expressed genes between the young and old mouse groups. Functional annotation analysis revealed 10 important pathways, including arachidonic acid metabolism and glutathione metabolism, involved in uterine cellular proliferation and decidualization. Using *in silico* analysis, we identified the three most abundantly interacting microRNAs–miR-223–3p, 155–5p, and 129–5p–with differentially expressed genes associated with important biological pathways. Samul-tang administration restored the expression of miR-223–3p, which could interact with important genes such as histamine ammonia-lyase (*Hal*) and acid phosphatase 5 (*Acp5*) for embryo implantation.

Conclusion: Our study demonstrated that uterine aging changes the expression of genes involved in metabolic pathways that are crucial to uterine health, leading to cellular senescence. We postulate that the regulation of miR-223–3p via Samul-tang administration can be of therapeutic importance in cellular senescence.

1. Introduction

The human endometrium undergoes cyclic changes in preparation for implantation of a blastocyst and maintenance of pregnancy if implantation occurs.[1] The uterus has a multicellular system comprising stromal, epithelial, vascular, and immune cells under the influence of complex biological signals.[2] Dynamic cell-to-cell dialog is essential for immunity, angiogenesis, tissue remodeling, and repair of the endometrium during the menstrual/estrus cycle.[3]

In humans, endometrial proliferation is initially promoted by estradiol in the proliferative phase of the menstrual cycle, and the endometrium thickens throughout this phase until ovulation.[4] After ovulation, progesterone controls the endometrium through several spiral arteries and glandular epithelial cells, which are characterized by abundant endoplasmic reticulum and the accumulation of glycogen-rich vacuoles.[5] The early secretory stage of the menstrual cycle in humans is homologous to the metestrus phase of the mouse estrous cycle.[6] During this phase of the menstrual/estrus cycle, endometrial decidualization spontaneously initiates to be receptive to blastocyst implantation under high levels of estrogen and progesterone. However, senescence of the uterine endometrium is accompanied by impaired decidualization, leading to decreased embryo receptivity and implantation failure.[7]

Uterine aging is characterized by the loss of function of steroid hormone activity, morphological changes in the uterine epithelium, accumulation of collagen fibrils in the uterine stroma, and impairment of decidual response.[8] Microarray analysis has shown that aging of the uterus leads to alterations in the expression of genes associated with cell proliferation in mice, indicating that senescent cells undergo cell proliferation arrest.[9, 10] In addition, microRNAs (miRNAs), small non-coding RNAs, and regulatory RNAs play critical roles in various biological events, including cellular

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senescence.[11] Mouse uterine transcriptome analysis has revealed mRNA expression and epigenetic changes that provide biological insights into the functional changes that occur in the cycling mouse uterus.[12] Nonetheless, the underlying mechanism of age-related transcriptomic changes in the endometrium during the estrous phase is unclear. Previously, we reported that Samul-tang (SM), comprising *Paeonia lactiflora, Ligusticum striatum, Rehmannia glutinosa*, and *Angelica giga*, improves implantation potential in aged mice.[13] This herbal preparation has been traditionally used to manage infertility symptoms in humans and rodent models.[14, 15] In this study, we explored uterine transcriptomic changes in the metestrus phase of the estrous cycle in the aged and hormonally stimulated uteri of mice. Furthermore, the changes in SM-treated aged mouse uteri were determined using *in silico* analysis.

2. Methods

2.1. Mice

All experiments and analyses were conducted in accordance with the relevant guidelines and regulations. The animal experimental protocols were approved by the Institutional Animal Care and Use Committee of the Korea Institute of Oriental Medicine, Daejeon, Korea (approval number 20–090). Female BALB/c mice aged 8 weeks (young, $n = 6$) and 40 weeks (old, $n = 6$) (Central Lab Animal Inc., Seoul, Korea) were housed under specific pathogen-free conditions and orally administered either distilled water or 2.5 g/kg SM (Hanpoong, Iksan, Korea) five times per week for 4 weeks. The mice were administered 5 IU of pregnant mare serum gonadotropin (Prospec, Rehovot, Israel) and 5 IU of human chorionic gonadotropin (Prospec) to mimic the gonadotropin releasing hormone-stimulated endometrial cycle before embryo transfer in *in-vitro* fertilization patients. The hormonally stimulated uteri from the mice were removed and immediately placed in liquid nitrogen until mRNA sequencing.

2.2. RNA sequencing for mRNA expression

We performed QuantSeq 3′mRNA sequencing to compare the mRNA expression patterns in the uterus of young and old mice in the metestrus phase of the estrous cycle. The hormonally stimulated uteri from 12- and 44-week-old mice corresponding to 20 and 40 years of women, respectively,[16, 17] were collected from the mice post-ovulation, and the total RNA was extracted from them using TRIzol™ (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The purity and integrity of the extracted RNA were evaluated using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and an Agilent 2100 bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands), respectively. All samples showed high purity (optical density (OD)260/OD280 $>$ 1.80) and integrity (RNA integrity number $>$7.0). RNA sequencing was performed using the Illumina NextSeq 500 platform. A fold-change value of $>$2.0 and P value of $<$0.05 were used as thresholds to identify differentially expressed genes (DEGs).

2.3. Functional annotation analysis of differentially expressed mRNAs

Gene enrichment and functional annotation analyses for the significant probe list were performed using the Gene Ontology (GO; http://geneontology.org) and Kyoto Encyclopedia of Genes and Genomes (KEGG; http://kegg.jp) databases to identify the potential functions of DEGs in biological pathways. Data analysis and visualization of DEGs were conducted using R 3.5.1 (www.r-project.org). A protein–protein interaction network of DEGs was constructed using the Search Tool for the Retrieval on Interacting Genes (STRING; http://string-db.org, version 11.0) database.

2.4. In silico prediction of DEG–miRNA networks with differentially expressed mRNAs

The target miRNAs were identified using miRNet (http://www.mirnet.ca/), a comprehensive tool that integrates data from 11 different miRNA databases (TarBase, miRTarBase, miRecords, miRanda, miR2Disease, HMDD, PhenomiR, SM2miR, PharmacomiR, EpimiR, and starBase).[18, 19]

2.5. Validation of the predicted target miRNAs in the uterus

To identify possible direct mRNA–miRNA interactions, the expression of seven miRNAs was confirmed using real-time qPCR. The miRNAs were reverse transcribed using the TaqMan microRNA Reverse Transcription kit and the relevant miRNA-specific stem-loop primers (Applied Biosystems, MA, USA) according to the manufacturer's protocol. The reverse transcription reaction conditions were as follows: 30 min at 16 °C for primer annealing, 30 min at 42 °C for primer extension, and 5 min at 85 °C for stopping the reaction. PCR was performed in a final reaction volume of 15 μL using the QuantStudio 6 Flex Real-time PCR System with fluorescent probe-based TaqMan small RNA assays according to the manufacturer's instructions (Thermo Fisher Scientific). The cycle threshold was normalized and compared using sno202 as the internal standard.

2.6. Statistical analysis

Data are presented as mean ± SEM. The statistical significance of the differences between the groups was determined using Student's *t*-test with GraphPad Prism version 8.4.0 (GraphPad Software, Inc., La Jolla, CA, USA). Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Profiling of age-related mRNA expression in aged mouse uteri

The hierarchical clustering analysis revealed that 4212 genes displayed significantly different expression levels in young and old mice (Fig. 1A). Among the 4212 genes, the volcano plot revealed 586 marked DEGs with a fold change $>$ 2.0 ($P < 0.05$; Fig. 1B). The 586 DEGs included 304 upregulated genes (51.9%) and 282 downregulated genes (48.1%), as shown in Supplementary Tables 1 and 2, which were analyzed using bioinformatic tools.

The KEGG pathway analysis of the young and old mouse datasets revealed that the DEGs are involved in arachidonic acid metabolism; glutathione metabolism; cytokine–cytokine receptor interaction; hematopoietic cell lineage; calcium signaling pathway; renin–angiotensin system; glycine, serine, and threonine metabolism; metabolic pathways; taurine and hypotaurine metabolism; and lysosomal metabolism (Table 1). We constructed a network of the DEGs using STRING (Fig. 2).

3.2. Identification of potential mRNA–miRNA regulatory network in aged mouse uterus

Target miRNAs of DEGs associated with the 10 pathways identified in the KEGG pathway analysis were predicted; they are summarized in Table 2. We identified the possible direct mRNA–miRNA interactions using miRNet (http://www.mirnet.ca/). We found seven miRNAs, namely miR-223–3p, miR-122–5p, miR-155–5p, miR-205–5p, miR-188–5p, miR-124–3p, and miR-129–5p, which

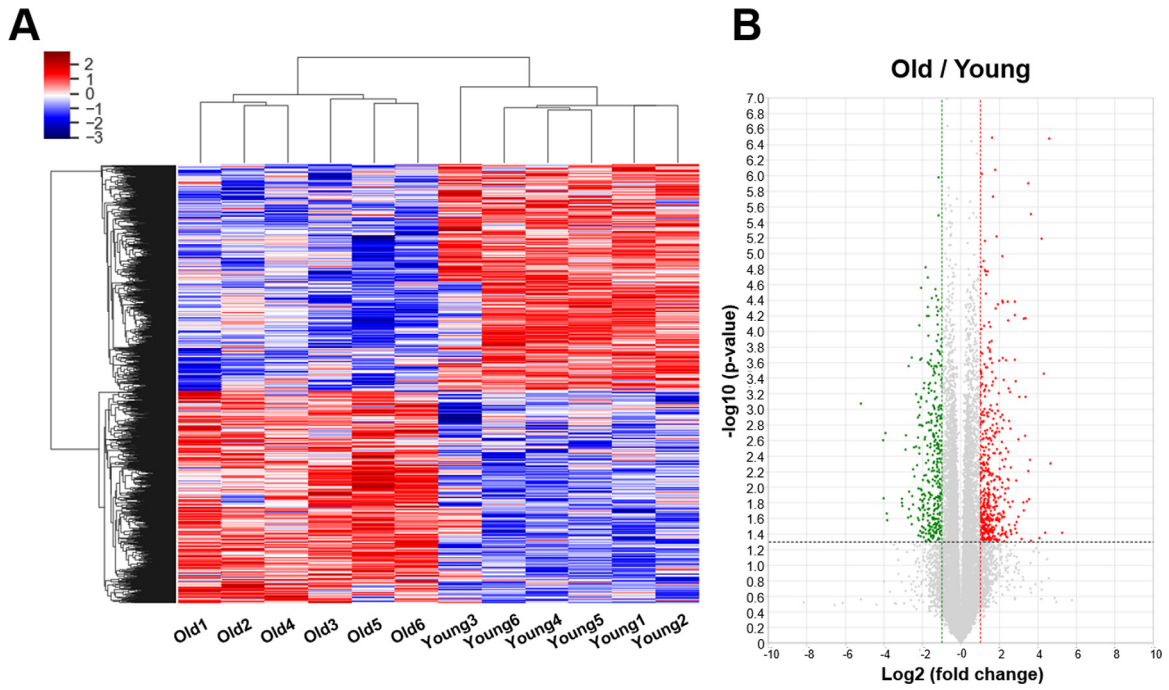

Fig. 1. Hierarchical clustering and analysis of differentially expressed mRNAs. Quantseq 3′mRNA analysis comparing gene expression profiles in the uterus of young ($n = 6$) and old ($n = 6$) mice in the metestrus phase of estrous cycle. (A) Hierarchical clustering among the mRNA expression profiles showing 4212 genes displaying significantly different expression levels in young and old mouse groups. (B) Volcano plot of 586 differentially expressed mRNAs between young and old mice with fold-change > 2.0 and $P < 0.05$. Red and green dots denote upregulated and downregulated genes, respectively. Young, 12-week-old control mice; Old, 44-week-old mice.

Table 1
List of genes involved in the 10 KEGG pathways identified in the Kyoto Encyclopedia for Genes and Genomes analysis.

Term	P-value	Genes
mmu00590:Arachidonic acid metabolism	<0.0001	*Cbr2, Ggt5, Hpgds, Gpx2, Pla2g2d, Gpx3, Alox12e, Alox15, Alox12, Ggt1, Ptgds*
mmu00480:Glutathione metabolism	<0.0001	*Ggt5, Gpx2, Gstm2, Ggt6, Gpx3, Anpep, Ggt1, Gstm7, Hpgds*
mmu04060:Cytokine-cytokine receptor interaction	<0.0001	*Cxcl9, Csf3, Ccl11, Csf1, Tnfrsf9, Cxcr4, Cxcr6, Cxcl13, Il17rb, Tnfsf13b, Bmp2, Ackr3, Cd27, Ccr7, Ltb, Il7r, Bmpr1b*
mmu04640:Hematopoietic cell lineage	0.0068	*Cd2, Csf3, Csf1, Mme, Anpep, Cd7, Cd3e, Il7r*
mmu04020:Calcium signaling pathway	0.0077	*Slc8a3, Ptgfr, Gna15, P2rx2, Erbb4, Nos3, Sphk1, Itpr2, Adrb1, Tacr1, Avpr1a, Nos1*
mmu04614:Renin-angiotensin system	0.0128	*Klk1b21, Klk1, Mme, Anpep, Klk1b24*
mmu00260:Glycine, serine and threonine metabolism	0.0202	*Aoc2, Sds, Cbs, Shmt1, Phgdh*
mmu01100:Metabolic pathways	0.0284	*Ippk, Galnt13, Shmt1, Hdc, Alox15, Alox12, Adh7, Fut4, Papss2, Hsd11b1, Hpgds, Cyp26b1, Sptlc3, Amdhd1, Hyal1, Cbs, Anpep, Alox12e, Lipg, Gcnt4, Phgdh, Nos1, Ggt1, Ptgd2, Ggt5, Cbr2, Pla2g2d, Pcyt1b, Sds, Ggt6, Aoc2, Gch1, Nos3, Sphk1, Gfpt2, Hal, Bdh1, P4ha3, B3gnt5, Piga, Aldh1as, Etnk1, Aldh1a1, Ada, Fbp2, Acp5*
mmu00430:Taurine and hypotaurine metabolism	0.0323	*Ggt5, Ggt6, Ggt1*
mmu04142:Lysosome	0.0413	*Napsa, Acp5, Ctsw, Ap1s3, Ctsd, Ap3b2, Ctsc, Cln5, Hyal1*

Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed the potential signaling pathways with which the differentially expressed genes between young and old mice were associated.

showed substantial interactions with the DEGs associated with important biological pathways (Fig. 3).

3.3. Changes in miRNA expression in aged mouse uteri following SM administration

The expression of the seven miRNAs in the aged mouse uteri was confirmed by qPCR using the fluorescent probe-based TaqMan small RNA assays (Fig. 4). The expression of miR-223–3p and miR-129–5p was upregulated, whereas the expression of miR-155–5p was downregulated in the aged uteri. This result indicates that the three aforementioned miRNAs might be important regulators of uterine aging. The increased expression of miR-223–3p in aged uteri was restored by SM administration (Fig. 4). This result indicates that SM administration could affect age-related genetic changes via miR-223–3p and lead to improved implantation potential.

The results also showed that miR-223–3p could interact with 19 genes, which were enriched in the 10 KEGG pathways, as determined using miRNet. Especially, sequencing data revealed that the expression of histidine ammonia-lyase (*Hal*) and acid phosphatase 5 (*Acp5*) in old mice was restored to the levels in young mice with SM administration ($P < 0.05$; Table 3).

4. Discussion

Senescent cells progressively accumulate and cause a gradual loss of organ function during aging.[20] This loss of functional properties leads to the loss of tissue homeostasis, which is linked to increased disease susceptibility.[21] Aging is also accompanied by biological mechanisms such as inflammation, metabolic dysfunction, and epigenetic changes.[22] An analysis of gene expression using microarray and sequencing technology would provide biological insights to help determine the regulatory targets that are crucial to aging. We discussed the endometrial cycle with an emphasis on

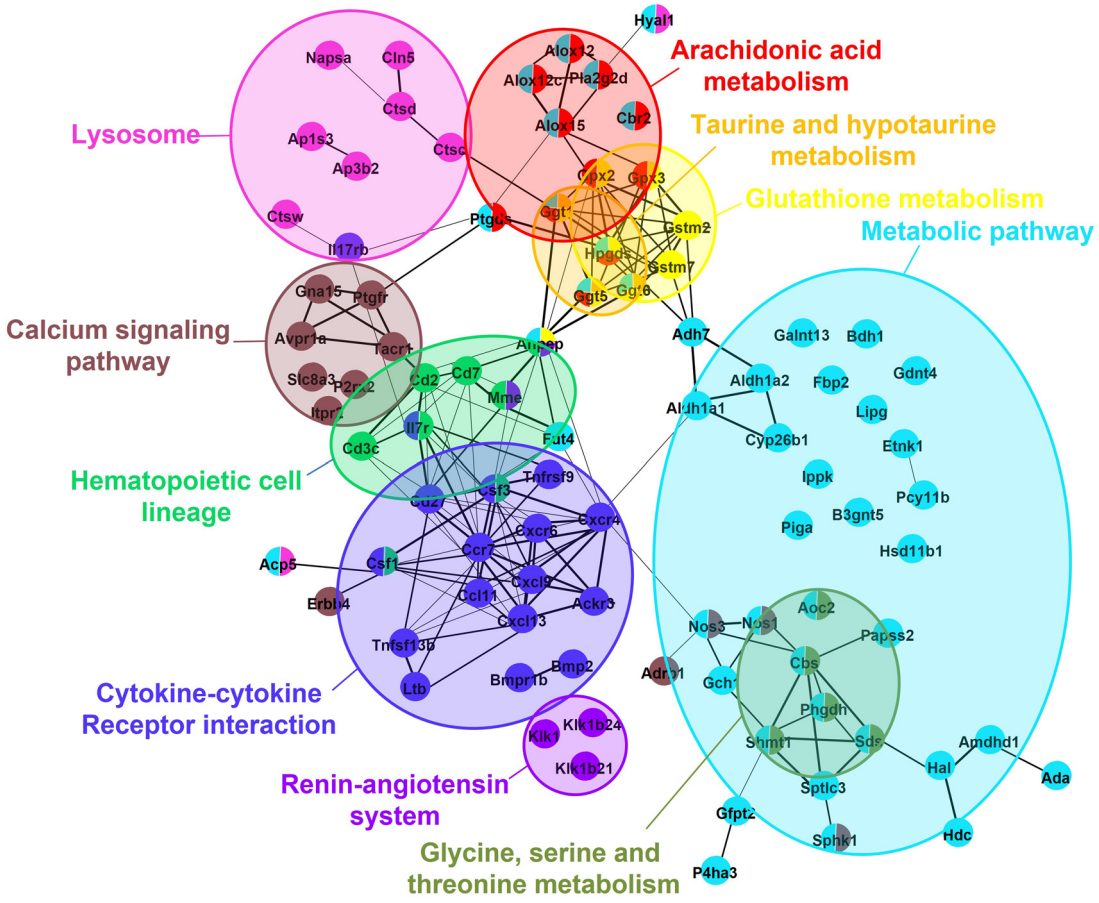

Fig. 2. Genetic regulatory network of differentially expressed genes. Protein–protein interaction network of the identified differentially expressed genes (DEGs) constructed using Search Tool for the Retrieval on Interacting Genes (STRING). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed that the DEGs are involved in arachidonic acid metabolism; glutathione metabolism; cytokine–cytokine receptor interaction; hematopoietic cell lineage; calcium signaling pathway; renin–angiotensin system; glycine, serine and threonine metabolism; metabolic pathways; taurine and hypotaurine metabolism; and lysosomal metabolism.

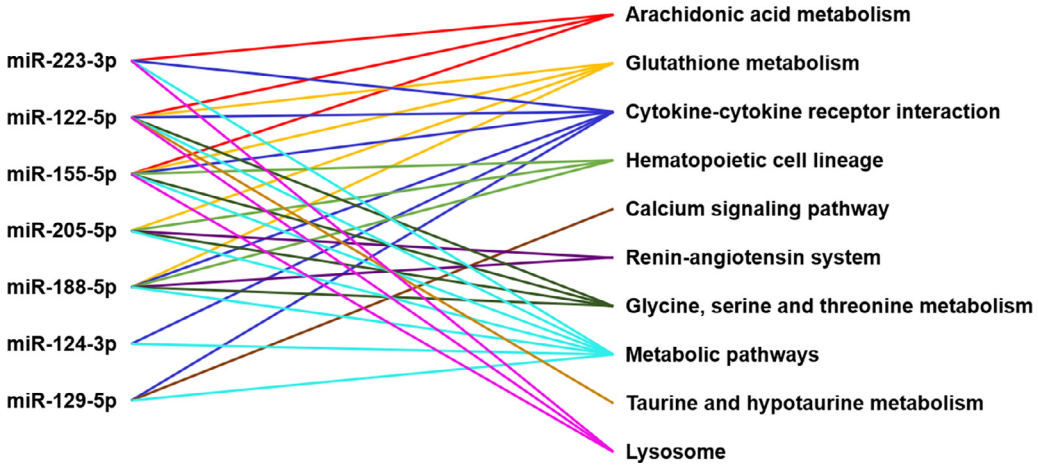

Fig. 3. microRNAs most abundantly interacting with genes involved in the 10 pathways identified in the Kyoto Encyclopedia for Genes and Genomes pathway analysis. The mRNA–miRNA interaction was identified using miRNet (http://www.mirnet.ca/).

the features of the metestrus phase, including genetic regulation of the uterus in aged mice.

Aged uteri in the early secretory phase undergo a dynamic change in the expression of a large number of genes involved in various metabolic pathways. While their proliferative potential decreases, senescent cells display high metabolic activity to acquire a more glycolytic state.[23] Cell senescence contributes to metabolic disturbances under hypoxic conditions.[24, 25] Glutathione metabolism plays an important role in antioxidant defense, nutrient metabolism, cellular proliferation regulation, cytokine production, and gene expression.[26] It is also related to aging and the pathogenesis of various diseases and is associated with an imbalance between the production and removal of reactive oxygen species.[27] Arachidonic acid is the most abundant fatty acid, and its metabolites play roles in diseases affecting fertility and pregnancy pathologies.[28] Arachidonic acid metabolism is normally observed

Table 2
List of microRNAs interacting with the differentially expressed genes involved in 10 pathways identified in the Kyoto Encyclopedia for Genes and Genomes analysis.

Term	miRNAs
mmu00590:Arachidonic acid metabolism	miR-223–3p, miR-122–5p, miR-155–5p, miR-1186b
mmu00480:Glutathione metabolism	miR-122–5p, miR-155–5p, miR-205–5p, miR-188–5p
mmu04060:Cytokine-cytokine receptor interaction	miR-223–3p, miR-122–5p, miR-155–5p, miR-188–5p, miR-124–3p, miR-129–5p, miR-27a-3p, miR-9–5p
mmu04640:Hematopoietic cell lineage	miR-155–5p, miR-205–5p, miR-188–5p
mmu04020:Calcium signaling pathway	miR-129–5p, miR-22–3p, miR-1a-1–5p, miR-1a-3p, miR-362–3p, miR-322–5p, miR-466i-5p
mmu04614:Renin-angiotensin system	miR-205–5p, miR-188–5p
mmu00260:Glycine, serine and threonine metabolism	miR-122–5p, miR-155–5p, miR-205–5p, miR-188–5p
mmu01100:Metabolic pathways	miR-223–3p, miR-122–5p, miR-155–5p, miR-205–5p, miR-188–5p, miR-124–3p, miR-129–5p, miR-1186b, miR-9–5p, miR-1a-1–5p, miR-1a-3p, miR-362–3p, miR-322–5p, miR-466i-5p, miR-125b-5p, let-7 g-5p, let-7c-5p, miR-101a-3p, miR-26a-5p, miR-340–5p, let-7b-5p, miR-29b-3p, miR-3473c, miR-17–5p, miR-193b-3p, miR-365–3p, miR-294–3p, miR-1187, miR-301b-3p, miR-16–5p, miR-34c-5p, miR-10b-5p, miR-3110–5p, miR-343, miR-692, miR-298–5p, miR-466l-5p, miR-669k-3p, miR-669n, miR-129–1–3p, miR-19b-3p, miR-324–3p, miR-712–5p, miR-338–3p, miR-143–5p, miR-671–5p, miR-320–3p miR-122–5p
mmu00430:Taurine and hypotaurine metabolism	miR-223–3p, miR-122–5p, miR-155–5p, miR-1a-3p, miR-882
mmu04142:Lysosome	

The seven most abundantly interacting miRNAs with differentially expressed genes involved in 10 KEGG pathways were identified using miRNet (http://www.mirnet.ca/).

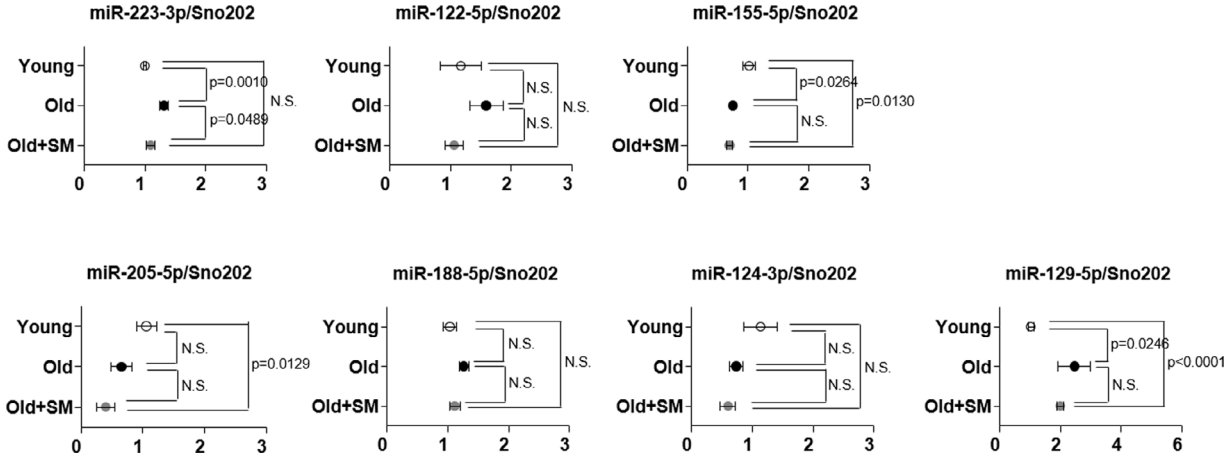

Fig. 4. Validation of the expression of seven predicted microRNAs interacting with the differentially expressed genes involved in the 10 pathways identified in the Kyoto Encyclopedia for Genes and Genomes pathway analysis. Validation of the expression of miR-223p-3p, miR-122–5p, miR-155–5p, miR-205–5p, miR-188–5p, miR-124–3p, and miR-129–5p through qPCR. Significant differences between the groups were calculated using Student's *t*-test. Data are presented as mean ± SEM. Young, 12-week-old control mice; Old, 44-week-old mice; Old+SM, 44-week-old mice orally administered Samul-tang.

in the endometrium during the proliferation and secretory phases of the menstrual cycle in humans.[29] Arachidonic acid and lipoxygenase metabolites play important roles in hormone signal transduction, decidual cell differentiation and the vascular function in the uterus.[30-32] Therefore, several metabolic pathways are crucial to uterine health. Aging-induced abnormalities in antioxidant and lipid metabolism might impair the endometrial microenvironment, leading to abnormal endometrial decidualization and implantation failure.

Epigenetic modifications, including DNA methylation and histone and RNA modifications, can cause several aging-related diseases such as degenerative disease and infertility.[33] The miRNAs act as epigenetic modulators; mammalian miRNAs control the post-transcriptional expression of their target genes by reg-

Table 3
List of genes interacting with miR-233–3p identified using the miRNet database.

Genes	Relative mRNA expression levels (log2)			p-value	
	Young	Old	Old+SM	Old vs Young	Old vs Old+SM
Ap1s3	7.713	9.300	8.916	0.038	0.454
Hal	5.242	7.105	6.270	0.002	0.036
Alox12	5.528	6.901	6.780	0.018	0.726
Alox12e	6.779	10.279	10.039	0.006	0.668
Alox5	6.256	10.927	10.465	0.005	0.413
Crt7	4.287	2.173	2.481	0.020	0.633
Aoc2	5.754	4.105	4.492	0.000	0.172
Gch1	6.117	5.050	5.324	0.012	0.284
Il7r	4.288	5.738	5.315	0.006	0.178
B3gnt5	4.952	5.997	5.505	0.035	0.193
Lipg	4.038	5.427	4.906	0.046	0.245
Aldh1a1	6.082	7.381	7.152	0.000	0.267
Nos3	8.135	7.030	7.330	0.000	0.354
Cxcl9	2.917	5.172	4.963	0.005	0.571
Hpgds	11.385	10.142	10.516	0.019	0.447
Etnk1	10.041	11.465	10.807	0.023	0.169
Tnfsf13b	5.179	6.407	6.190	0.001	0.239
Fut4	5.911	4.831	4.449	0.038	0.458
Acp5	7.815	6.719	7.557	0.008	0.002

The webtool miRNet identified that the miR-223–3p interacts with 19 genes involved in 10 KEGG pathways (http://www.mirnet.ca/). Young, 12-week-old control mice; Old, 44-week-old mice; Old+SM, 44-week-old mice orally administered Samul-tang.

ulating the translation or stability of mRNAs.[34] Through mRNA–miRNA regulatory network analysis, we identified the seven most abundant miRNAs interacting with genes involved in the 10 pathways identified in the KEGG pathway analysis. Several studies have shown the role of miRNAs in aging and endometrial pathogenesis. Three miRNAs, namely miR-122–5p, miR-155–5p, and miR-188–5p, have been reported as peripheral biomarkers in aging and age-related diseases.[35-37] miR-205–5p and miR-129–5p inhibit cancerous behaviors by regulating endometrial stromal cell migration and apoptosis in humans.[38, 39] Here, SM administration induced a decrease in miR-223–3p expression, which has an inhibitory effect on leukemia inhibitory factor (*Lif*) and pinopode formation.[40] LIF and its receptor play a key role in successful embryo implantation.[41] Furthermore, the expression of *Hal* and *Acp5* in the uterus of old mice was restored to the levels in young mice with SM administration. The decrease in *Hal* expression could lead to an increase in histamine production by increasing histidine. Histamine produced by uterine epithelial cells is one of well-known mediators for embryo–uterine interactions during implantation.[42, 43] *Acp5*, an evolutionarily conserved gene in the endometrium, is considered to perform an important role in pregnancy.[44] Therefore, we suggest that the regulation of miR-223–3p by SM might be of therapeutic importance in cellular senescence and fertility. Although *P. lactiflora*, one of the medical herbs constituting Samul-tang, could promote implantation by inducing LIF expression, the relationship between *P. lactiflora* and miR-223–3p has not been evaluated.[45] Therefore, it is necessary to find the effective functioning compounds among the components of Samul-tang. The roles of the aforementioned miRNAs in the cellular and molecular processes of uterine aging need to be further investigated.

Here, the uterine transcriptome analysis of reproductively aged mice revealed mRNA expression changes that provided biological insights into the functional changes in aging mouse uteri during the metestrus phase of the estrous cycle. We investigated the age-related epigenetic modifications in the uterus of aged mice during the metestrus phase of the estrous cycle. However, hormonal stimulation of the uterus could influence endometrial cellular proliferation and uterine function.[46] Functional analysis of DEGs and miRNAs is still needed to develop efficient strategies and approaches for preventing uterine aging-associated problems, including defects in uterine receptivity.[47] In conclusion, uterine aging changed the expression of genes involved in metabolic pathways, leading to cellular senescence. SM restored the expression of genes involved in implantation potential in aged uteri, but further investigations are needed. Consideration of SM-induced genetic interactions may help improve poor uterine environment or repeated implantation failure in aged patients.[48]

Author contributions

Conceptualization: J Kim. Investigation: J Kim, S You. Writing – Original Draft: J Kim. Writing – Review & Editing: J Kim, S You. Supervision: S You. Project administration: S You. Funding acquisition: S You.

Conflict of interest

The authors have no conflict of interests to declare.

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Ethical statement

This research was approved by the institutional animal care and use committee of the Korea Institute of Oriental Medicine (approval number 20-090).

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.imr.2022.100835.

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