Title

Puerarin blocks aging phenotype in cultured human dermal fibroblasts

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18 Abstract

19 Dermal fibroblast aging contributes to aging-associated functional defects in the skin
 20 since dermal fibroblasts are important to maintain skin homeostasis by interacting with
 21 epidermis and extracellular matrix. Here we identified that puerarin, an isoflavone contained
 22 in *Pueraria lobata* (Kudzu), can prevent the aging-phenotype of human dermal fibroblasts.
 23 Puerarin treatment increased in proliferating cells and decreased in senescence-associated beta-
 24 galactosidase positive cells in the high-passage culture of dermal fibroblasts. Moreover, puerarin
 25 reduced smooth muscle actin-positive myofibroblasts and the expression of a reticular fibroblast
 26 marker, calponin 1 (CNN1), which were induced in high-passage fibroblasts. Fulvestrant, an
 27 estrogen receptor antagonist, blocked puerarin-mediated downregulation of SMA and CNN1.
 28 Our results suggest that puerarin may be a useful food factor that alleviates aging-related
 29 functional defects in the skin.

31 Keywords

32 Dermal fibroblast, senescence, myofibroblast, puerarin

34 Abbreviations

35 NHDFs; normal human dermal fibroblast, SMA; smooth muscle actin, CNN1; calponin 1, ER;
 36 estrogen receptor

37 Introduction

38 Skin aging is dependent on the diet and nutrition as well as the other intrinsic factors,
39 such as chronological aging, and the extrinsic factors, such as sun exposure [1,2]. Since excessive
40 reactive oxygen species (ROS) generated by the harmful stresses cause cellular senescence and
41 aging phenotype, anti-oxidative rich foods have been well investigated as a preventive strategy
42 against skin aging [2–4]. Additionally, recent evidence suggests that targeting aging-associated
43 changes by natural products can prevent age-related phenotype.

44 Cellular senescence is a hallmark of normal aging, which is a state of irreversible growth
45 arrest triggered by both endogenous stress and exogenous stress [5–7]. Senescent cells are
46 characterized by the induction of senescence-associated beta-galactosidase activity, telomere
47 shortening, the expression of cyclin-kinase inhibitors, such as p16 or p21, etc. Senescent cells
48 secrete proinflammatory cytokines, growth factors, and matrix metalloproteases, that
49 contributes to local and systemic dysfunction [8,9]. Accumulating evidence demonstrated that
50 clearance of senescent cells prevents the age-associated decline of tissue function and extends
51 lifespan [10]. Recently, small molecules, including the nutritional agents, that can selectively
52 eliminate senescent cells, called senolytics, are expected to prevent age-associated decline of
53 tissue function[7,11,12].

54 Fibroblasts play an important role in maintaining tissue structure by the synthesis of
55 extracellular matrix proteins, such as collagen and elastin, in most tissues. The skin dermis
56 has three layers, the papillary dermis, the reticular dermis and the hypodermis [13]. The
57 papillary dermis is the superficial layer that contacts the epidermis and has a higher cell density,
58 while the reticular dermis is the deeper layer and has a thicker extracellular matrix and a lower
59 cell density [14,15]. The fibroblasts residing in papillary and reticular dermis have distinct
60 identities. Papillary fibroblasts exhibit a spindle-shaped morphology and show higher
61 proliferative activity than reticular fibroblasts. Meanwhile, reticular fibroblasts exhibit a

polygonal morphology and express the myofibroblast marker alpha-smooth muscle actin [16]. Aging mainly affects the cellular function of papillary fibroblasts, including a decrease in proliferative capacity and an increase in transdifferentiation to reticular fibroblasts, which may lead the aging-associated structure changes [17–20]. Recent studies showed that diet and nutrition can influence the skin phenotype by controlling the aging of dermal fibroblast [21–23], proposing that nutritional agents that modify the fibroblast function links skin aging.

Puerarin (daidzein-8-C-glucoside) is a major isoflavone found in *Pueraria lobata* (Kudzu, Kuzu, Gegen) which is an edible legumes [21–24]. Puerarin is abundant in vine and root of *Pueraria lobata*; 0.1 ~ 1 g in 100 g of the vine, 3 ~ 10 g in 100g of the root [25–28]. The root of *Pueraria lobata* is also used as a component of traditional Chinese medicine to treat chills, colds, fever, and diarrhea. Recent studies revealed a variety of pharmacological actions of puerarin, such as vasodilatation, cardioprotection, anticancer, menopausal symptoms, etc. [29,30]. Oral ingested puerarin is absorbed from the gut into blood; the oral bioavailability of puerarin has been reported to be approximately 4-7% in animal models [31–34]. Concerning the effects on aging, puerarin reduces the endothelial progenitor cell senescence [35]. However, the effects of puerarin on skin aging and fibroblast phenotype remain to be elucidated.

In the present study, we investigated the cytoprotective and anti-aging properties of puerarin in the normal human dermal fibroblasts in vitro.

Methods

Cell culture

Normal human dermal fibroblasts (PromCell, Heidelberg, Germany) were cultured in DMEM containing 10% fetal bovine serum and antibiotics (penicillin and streptomycin, Nacalaitesque Kyoto, Japan) according to the previous report [17]. Cells were subcultivated with 1:2 to

1:5 split ratio every week. High-passage fibroblasts (25-35 passages) were confirmed to have lower proliferative activity and were used as senescent cells. Low-passage fibroblasts (5-15 passages) were used as young control. Before experiments, NHDFs were plated on the 96-well plate or 6-well plate, then were treated with DMSO (1:1000) or puerarin (25-50 μ M) and Fulvestrant (1 μ M, [36]) next day, and were cultured for more 72 h.

Cell viability and cell injury assays

NHDFs were plated on the 96-well plate at 2,500 - 10,000 cells per well, and then were cultured with puerarin. Cell viability and cell toxicity were evaluated by Cell counting kit-8 assay and lactate dehydrogenase (LDH) assay (Dojindo, Kumamoto, Japan), respectively, according to the manufacturer's protocols.

Beta-Garactosidase activity

The Senescence-associated (SA) beta-galactosidase activity was detected as previously reported [37]. In brief, after fixed by 10% formalin in PBS, cells were incubated in X-gal stain solution (5mM potassium ferricyanide, 5mM potassium ferrocyanide, 2mM $MgCl_2$ and 0.5 mg/mL X-gal in citric acid/potassium phosphate buffer (pH 6.0)) at 37 °C overnight. The numbers of the stained cells per microscope field of view (x100) were calculated as the senescent cells.

BrdU assay

BrdU assay was performed as previously reported [38] with brief modification. NHDFs were plated on the 96-well plate at 5,000 cells per well. After cultured with or without puerarin, BrdU was added to the culture medium and the cells were cultured for more 24 h. After fixed by 10% formalin in PBS and then incubated with 2 mol/L HCl for 10 min at room temperature and for 20 min more at 37°C to denature DNA, BrdU incorporated in DNA was detected by the

immunocytochemical method. The numbers of BrdU-positive cells per microscope field of view (x100) were calculated.

Measurement of intracellular ROS

After cells on the 96-well plate were cultured with or without puerarin for 72 h, CellROX Green reagent (final concentration: 5 μ M, ThermoScientific, USA) was added into the culture medium, and incubated for more 30 min. Then cells were washed and fixed with 10% formalin in PBS, and cells were observed by fluorescent microscopy. The fluorescent intensity was determined by using ImageJ software.

mRNA measurement

Total RNA was isolated from NHDFs by using FastGene™ RNA Basic Kit (Fast Gene, Japan) and cDNA was generated by using Rever Tra Ace (Toyobo, Osaka, Japan). Quantitative RT-PCR analysis was performed by using Applied Biosystems Step One (Applied Biosystems) with THUNDERBIRD SYBR qPCR Mix (Toyobo) according to the previous report [39]. The primers used are shown in Table 1. To consider the specificity of the primer set for SMA, a set of primers which has been designed to selectively detect the human SMA was used [40]. The primers for the other genes were originally designed by using Primer-BLAST tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>).

Immunofluorescence microscopic examination

After NHDFs were fixed by 10% formalin in PBS and then were permeabilized by 0.1 % Triton-X 100 in PBS for 10 min, and blocked in BlockingOne reagent (Nacalai tesque, Kyoto, Japan). Immunostaining was performed by using specific primary antibodies and the fluorescent secondary antibodies diluted in Can Get Signal Immunostain (Toyobo) according to the previous report [41]. Nuclear were stained with DAPI. Cells were examined by Fluorescent

microscopy (Leica). Antibodies used in this study are as follows; Mouse anti- α -SMA actin IgG (Abcam), Rat anti-BrdU IgG (Abcam), Alexa 555-conjugated goat anti-mouse IgG antibody (Thermoscientific), CF488-conjugated anti-rat IgG antibody (Biotium).

Statistical analysis

Student's t-test or t-test with Bonferroni correction (for multiple comparisons) were used to determine statistical significance between groups. Differences were considered statistically significant when the calculated P-value was less than 0.05.

Results

The protective effects of puerarin in senescent NHDFs

To explore the effect of puerarin against skin aging, we tested the effect of puerarin on the cell viability in replication-induced senescence (Fig. 1). After passing (25~35 passages), the proliferation of NHDFs became slow. Treatment of puerarin (25-50 μ M) for 72h increased the cell viability of high passaged senescent NHDFs, although it did not influence in low passaged young NHDFs (Fig. 1A,B). Cell injury during normal culture, as measured by LDH release, was attenuated by puerarin treatment in senescent NHDFs (Fig. 1C). We investigated the effect of puerarin on the proliferation rate in high-passaged NHDFs, as assessed by BrdU assay (Fig. 1D,E). BrdU positive cells in microscopic fields were increased in puerarin-treated NHDFs, indicating that puerarin enhances cell proliferation.

Moreover, we evaluated the effect of puerarin on the ROS generation in senescent NHDFs, as assessed by the fluorescent ROS probe (Fig. 2). Since isoflavones possess an anti-oxidative role [36], we expected that puerarin lowers ROS production. However, puerarin treatment rather increased ROS production in senescent NHDFs.

162

163 **The anti-aging effects of puerarin in senescent NHDFs**

164 Next, we evaluated the effect of puerarin on the senescent phenotype of NDHF. SA-
165 beta-galactosidase positive NDHFs were decreased by puerarin treatment (Fig. 3A,B).

166 Another characteristic of aged dermal fibroblasts is a decrease in papillary fibroblasts and an
167 increase in the reticular fibroblasts in the skin [18,22]. In the case of the NHDF culture model,
168 high passage of NHDFs causes an increase in the cells of reticular phenotype and smooth muscle
169 actin (SMA)-positive myofibroblasts [19,42]. Therefore, we first evaluated the effect of puerarin
170 on the frequency of α -SMA-positive cells. Puerarin treatment reduced the numbers of α -SMA-
171 positive cells in high passaged senescent NHDFs (Fig. 4A). A reduction in SMA by puerarin
172 treatment was also validated by mRNA expression (Fig. 4B).

173 Next, we evaluated the effect of puerarin on the gene markers of papillary and reticular
174 phenotype, podoplanin (PDPN), and calponin-1 (CNN1), respectively (Fig. 5). Puerarin treatment
175 upregulated PDPN expression in senescent NHDFs. In contrast, puerarin treatment reduced
176 CNN1 expression in senescent NHDFs, while the CNN1 expression was increased by high
177 passage compared to low passage NHDFs, indicating that puerarin prevents the
178 transdifferentiation to reticular fibroblasts.

179

180 **The role of Estrogen receptor signaling pathway on the anti-aging effects of puerarin**

181 Since isoflavones possess an estrogen receptor (ER) agonist property, we tested whether
182 the ER pathway is involved in the effect of puerarin on the gene expression in NHDFs. An ER
183 antagonist, Fulvestrant, blocked puerarin-dependent downregulation of SMA and CNN1 (Fig. 6).
184 These data indicate that the attenuation of transdifferentiation to reticular fibroblasts by
185 puerarin may be associated with the ER pathway.

186

187 Discussion

188 In the present study, we evaluated the effect of puerarin in the senescent NHDFs and
189 discovered that puerarin enhances the viability of NHDFs accompanied by the increased
190 proliferative activity and the decreased cell injury. Furthermore, we found that puerarin
191 attenuates the frequency of SA-beta-galactosidase positive senescent cells. Puerarin also
192 attenuates aging-related fibroblast subpopulation changes; i.e. a decrease in myofibroblasts and
193 reticular fibroblasts by puerarin. Importantly, high passages cause to differentiate from papillary
194 fibroblasts into reticular fibroblasts, suggesting that puerarin may prevent age-related fibroblast
195 transdifferentiation.

196 While puerarin possesses a wide variety of pharmacological properties, various
197 molecular mechanisms have also been proposed [29]. Among them, puerarin is recognized as a
198 phytoestrogen. Puerarin or the extract of *Pueraria Lobata* has a beneficial effects on
199 menopausal symptoms, such as bone loss [25]. Whereas puerarin has a weak estrogenic activity,
200 puerarin also exerts an antagonistic effect against 17- β -estradiol and estrogen-response element-
201 reporter transcriptional activity [29]. In the present study, we found that ER inhibitor,
202 Fulvestrant, blocked the effect of puerarin on the SMA and CNN1 expressions, indicating that
203 the effects of puerarin on senescent NHDFs are via ER pathway. Since estrogens are key
204 hormones for skin aging homeostasis, and act directly on dermal fibroblasts [43,44], puerarin
205 might contribute to this mechanism.

206 Additionally, puerarin possesses antioxidant properties [29]. In the present study, we
207 found that ROS production was not decreased, rather increased in puerarin-treated NHDFs. In
208 the case of proliferation-induced senescent cells, excessive ROS production may not be associated
209 with cellular defects. In contrast, the appropriate generation of ROS has beneficial anti-aging
210 roles by modulating some signaling pathways [2]. Therefore, puerarin-induced ROS generation
211 may be involved in its anti-aging effect in NHDFs.

In the present study, we observed a reduction in the frequency of SA- β -gal positive cells by puerarin, suggesting the senolytic property of puerarin in NHDFs. However, we also observed that puerarin did not cause to induce cell toxicity in high-passaged NHDFs, as confirmed by LDH assay and the ethidium homodimer-I assay (live-dead assay, data not shown). Taken together, puerarin is not as a senolytics for senescent NHDFs.

Our present study indicates that the *Pueraria lobata* diet may confer anti-aging properties in the skin. Meanwhile, the bioavailability of orally ingested puerarin has been evaluated in rodents, and it is approximately 4-7% [34]. When 10 mg/ kg body weight of puerarin was administered to the rodents, Cmax of blood puerarin was 228 μ g/L (approximately 0.5 μ mol/L). In the present study, we confirmed the short-term effect of puerarin at 25 to 50 μ mol/L in the culture medium, and it is much higher than the blood concentration expected to be after the *Pueraria lobata* diet. Long-term and low-dose *Pueraria lobata* diet study will be necessary to confirm the clinical effect in the future.

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References

1. Zhang S, Duan E. Fighting against Skin Aging: The Way from Bench to Bedside. Cell Transplantation. SAGE Publications Ltd; 2018. pp. 729–738.
doi:10.1177/0963689717725755
2. Schagen SK, Zampeli VA, Makrantonaki E, Zouboulis CC. Discovering the link between nutrition and skin aging. Dermato-Endocrinology. Taylor & Francis; 2012. p. 298.
doi:10.4161/derm.22876
3. Iizaka S, Nagata S, Sanada H. Nutritional status and habitual dietary intake are

associated with frail skin conditions in community-dwelling older people. *J Nutr Heal Aging*. 2017;21: 137–146. doi:10.1007/s12603-016-0736-8

4. Mekić S, Jacobs LC, Hamer MA, Ikram MA, Schoufour JD, Gunn DA, et al. A healthy diet in women is associated with less facial wrinkles in a large Dutch population-based cohort. *J Am Acad Dermatol*. 2019;80: 1358-1363.e2. doi:10.1016/j.jaad.2018.03.033

5. Wang AS, Dreesen O. Biomarkers of cellular senescence and skin aging. *Front Genet*. 2018;9: 1–14. doi:10.3389/fgene.2018.00247

6. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. *Cell*. Cell Press; 2013. p. 1194. doi:10.1016/j.cell.2013.05.039

7. Li W, Qin L, Feng R, Hu G, Sun H, He Y, et al. Emerging senolytic agents derived from natural products. *Mechanisms of Ageing and Development*. Elsevier Ireland Ltd; 2019. pp. 1–6. doi:10.1016/j.mad.2019.05.001

8. Waldera Lupa DM, Kalfalah F, Safferling K, Boukamp P, Poschmann G, Volpi E, et al. Characterization of Skin Aging-Associated Secreted Proteins (SAASP) Produced by Dermal Fibroblasts Isolated from Intrinsically Aged Human Skin. *J Invest Dermatol*. 2015;135: 1954–1968. doi:10.1038/jid.2015.120

9. Coppé J-P, Desprez P-Y, Krtolica A, Campisi J. The Senescence-Associated Secretory Phenotype: The Dark Side of Tumor Suppression. *Annu Rev Pathol Mech Dis*. 2010;5: 99–118. doi:10.1146/annurev-pathol-121808-102144

10. Baker DJ, Childs BG, Durik M, Wijers ME, Sieben CJ, Zhong J, et al. Naturally occurring p16 Ink4a⁺ positive cells shorten healthy lifespan. *Nature*. 2016;530: 1–5. doi:10.1038/nature16932

11. Zhu Y, Tchkonina T, Pirtskhalava T, Gower AC, Ding H, Giorgadze N, et al. The Achilles' heel of senescent cells: from transcriptome to senolytic drugs. *Aging Cell*. 2015;14: 644–658. doi:10.1111/acer.12344

- 262 12. Xu M, Pirtskhalava T, Farr JN, Weigand BM, Palmer AK, Weivoda MM, et al. Senolytics
263 improve physical function and increase lifespan in old age. *Nat Med.* 2018;24: 1246–1256.
264 doi:10.1038/s41591-018-0092-9
- 265 13. Lynch MD, Watt FM. Fibroblast heterogeneity: implications for human disease. *Journal*
266 *of Clinical Investigation.* American Society for Clinical Investigation; 2018. pp. 26–35.
267 doi:10.1172/JCI93555
- 268 14. Janson DG, Saintigny G, Van Adrichem A, Mahé C, El Ghalbzouri A. Different gene
269 expression patterns in human papillary and reticular fibroblasts. *J Invest Dermatol.*
270 2012;132: 2565–2572. doi:10.1038/jid.2012.192
- 271 15. Philippeos C, Telerman SB, Oulès B, Pisco AO, Shaw TJ, Elgueta R, et al. Spatial and
272 Single-Cell Transcriptional Profiling Identifies Functionally Distinct Human Dermal
273 Fibroblast Subpopulations. *J Invest Dermatol.* 2018;138: 811–825.
274 doi:10.1016/j.jid.2018.01.016
- 275 16. Sriram G, Bigliardi PL, Bigliardi-Qi M. Fibroblast heterogeneity and its implications for
276 engineering organotypic skin models *in vitro.* *European Journal of Cell Biology.* Elsevier
277 GmbH; 2015. pp. 483–512. doi:10.1016/j.ejcb.2015.08.001
- 278 17. Brun C, Jean-Louis F, Oddos T, Bagot M, Bensussan A, Michel L. Phenotypic and
279 functional changes in dermal primary fibroblasts isolated from intrinsically aged human
280 skin. *Exp Dermatol.* 2016;25: 113–119. doi:10.1111/exd.12874
- 281 18. Mine S, Fortunel NO, Pigeon H, Asselineau D. Aging alters functionally human dermal
282 papillary fibroblasts but not reticular fibroblasts: A new view of skin morphogenesis and
283 aging. *PLoS One.* 2008;3. doi:10.1371/journal.pone.0004066
- 284 19. Janson D, Saintigny G, Mahé C, Ghalbzouri A El. Papillary fibroblasts differentiate into
285 reticular fibroblasts after prolonged *in vitro* culture. *Exp Dermatol.* 2013;22: 48–53.
286 doi:10.1111/exd.12069

- 287 20. Driskell RR, Lichtenberger BM, Hoste E, Kretzschmar K, Simons BD, Charalambous M,
288 et al. Distinct fibroblast lineages determine dermal architecture in skin development and
289 repair. *Nature*. 2013;504: 277–281. doi:10.1038/nature12783
- 290 21. Salzer MC, Lafzi A, Berenguer-Llergo A, Youssif C, Castellanos A, Solanas G, et al.
291 Identity Noise and Adipogenic Traits Characterize Dermal Fibroblast Aging. *Cell*.
292 2018;175: 1575–1590.e22. doi:10.1016/j.cell.2018.10.012
- 293 22. Lämmermann I, Terlecki-Zaniewicz L, Weinmüllner R, Schosserer M, Dellago H, de
294 Matos Branco AD, et al. Blocking negative effects of senescence in human skin
295 fibroblasts with a plant extract. *npj Aging Mech Dis*. 2018;4: 1–10. doi:10.1038/s41514-
296 018-0023-5
- 297 23. Forni MF, Pelligia J, Braga TT, Chinchilla JEO, Shinohara J, Navas CA, et al. Caloric
298 Restriction Promotes Structural and Metabolic Changes in the Skin. *Cell Rep*. 2017;20:
299 2678–2692. doi:10.1016/j.celrep.2017.08.052
- 300 24. Kirakosyan A, Kaufman PB, Warber S, Bolling S, Chang SC, Duke JA. Quantification of
301 major isoflavonoids and L-canavanine in several organs of kudzu vine (*Pueraria*
302 *montana*) and in starch samples derived from kudzu roots. *Plant Sci*. 2003;164: 883–888.
303 doi:10.1016/S0168-9452(03)00077-3
- 304 25. Tanaka T, Tang H, Yu F, Michihara S, Uzawa Y, Zaima N, et al. Kudzu (*Pueraria lobata*)
305 vine ethanol extracts improve ovariectomy-induced bone loss in female mice. *J Agric*
306 *Food Chem*. 2011;59: 13230–13237. doi:10.1021/jf2031617
- 307 26. Shimizu H. Isoflavonoids in the Stalk of *Pueraria lobata* Ohwi(3rd Report). *Rep Nara*
308 *Prefect Inst Ind Dev*. 2017; 30–32. Available: <http://ci.nii.ac.jp/naid/40021360790/en/>
- 309 27. MIKAGE M, MATSUMURA M, TAKAHASHI S. Studies of Chinese Crude Drug “Ge-
310 gen”. (II). Collection Season and Differences of Components. *Kampo Med*. 2002;53: 503–
311 507. doi:10.3937/kampomed.53.503

- 312 28. Chen G, Zhang J, Ye J. Determination of puerarin, daidzein and rutin in *Pueraria lobata*
313 (Wild.) Ohwi by capillary electrophoresis with electrochemical detection. *J Chromatogr*
314 A. 2001;923: 255–262. doi:10.1016/S0021-9673(01)00996-7
- 315 29. Zhou Y-X, Zhang H, Peng C. Puerarin: A Review of Pharmacological Effects. *Phyther*
316 *Res.* 2014;28: 961–975. doi:10.1002/ptr.5083
- 317 30. TANAKA T, YOKOTA Y, TANG H, ZAIMA N, MORIYAMA T, KAWAMURA Y. Anti-
318 Hyperglycemic Effect of a Kudzu (<i>Pueraria lobata</i>) Vine Extract in
319 Ovariectomized Mice. *J Nutr Sci Vitaminol (Tokyo)*. 2016;62: 341–349.
320 doi:10.3177/jnsv.62.341
- 321 31. Ma Z, Wu Q, Lee DYW, Tracy M, Lukas SE. Determination of puerarin in human plasma
322 by high performance liquid chromatography. *J Chromatogr B Anal Technol Biomed Life*
323 *Sci.* 2005;823: 108–114. doi:10.1016/j.jchromb.2005.06.016
- 324 32. Gao C, Li X, Li Y, Wang L, Xue M. Pharmacokinetic interaction between puerarin and
325 edaravone, and effect of borneol on the brain distribution kinetics of puerarin in rats. *J*
326 *Pharm Pharmacol.* 2010;62: 360–367. doi:10.1211/jpp.62.03.0011
- 327 33. Kong H, Wang X, Shi R, Zhao Y, Cheng J, Yan X, et al. Pharmacokinetics and Tissue
328 Distribution Kinetics of Puerarin in Rats Using Indirect Competitive ELISA. *Molecules*.
329 2017;22: 939. doi:10.3390/molecules22060939
- 330 34. Anukunwithaya T, Poo P, Hunsakunachai N, Rodsiri R, Malaivijitnond S, Khemawoot P.
331 Absolute oral bioavailability and disposition kinetics of puerarin in female rats. *BMC*
332 *Pharmacol Toxicol.* 2018;19: 25. doi:10.1186/s40360-018-0216-3
- 333 35. Sun C, Wang X, Zheng G, Fan S, Lu J, Zhang Z, et al. Protective effect of different
334 flavonoids against endothelial senescence via NLRP3 inflammasome. *J Funct Foods*.
335 2016;26: 598–609. doi:10.1016/j.jff.2016.08.031
- 336 36. Hwang YP, Jeong HG. Mechanism of phytoestrogen puerarin-mediated cytoprotection

following oxidative injury: Estrogen receptor-dependent up-regulation of PI3K/Akt and
HO-1. *Toxicol Appl Pharmacol.* 2008;233: 371–381. doi:10.1016/j.taap.2008.09.006

37. Debacq-Chainiaux F, Erusalimsky JD, Campisi J, Toussaint O. Protocols to detect
senescence-associated beta-galactosidase (SA- β gal) activity, a biomarker of senescent
cells in culture and in vivo. *Nat Protoc.* 2009;4: 1798–1806. doi:10.1038/nprot.2009.191

38. Takagi S, McFadden ML, Humphreys RE, Woda BA, Sairenji T. Detection of 5-bromo-2-
deoxyuridine (BrdUrd) incorporation with monoclonal anti-brdurd antibody after
deoxyribonuclease treatment. *Cytometry.* 1993;14: 640–648. doi:10.1002/cyto.990140608

39. Nguyen KH, Ito S, Maeyama S, Schaffer SW, Murakami S, Ito T. In Vivo and in Vitro
Study of N-Methyltaurine on Pharmacokinetics and Antimucle Atrophic Effects in Mice.
ACS Omega. 2020;5: 11241–11246. doi:10.1021/acsomega.0c01588

40. Veres-Székely A, Pap D, Sziksz E, Jávorszky E, Rokony R, Lippai R, et al. Selective
measurement of α smooth muscle actin: Why β -actin can not be used as a housekeeping
gene when tissue fibrosis occurs. *BMC Mol Biol.* 2017;18. doi:10.1186/s12867-017-0089-9

41. Ito T, Yoshikawa N, Ito H, Schaffer SW. Impact of taurine depletion on glucose control
and insulin secretion in mice. *J Pharmacol Sci.* 2015;129: 59–64.
doi:10.1016/j.jphs.2015.08.007

42. Baranyi U, Winter B, Gugerell A, Hegedus B, Brostjan C, Laufer G, et al. Primary
Human Fibroblasts in Culture Switch to a Myofibroblast-Like Phenotype Independently
of TGF Beta. *Cells.* 2019;8: 721. doi:10.3390/cells8070721

43. Verdier-Sevrain S, Bonte F, Gilcrest B. Biology of estrogens in skin: implications for
skin aging. *Exp Dermatol.* 2006;15: 83–94. doi:10.1111/j.1600-0625.2005.00377.x

44. Saville CR, Holmes DF, Swift J, Derby B, Emmerson E, Hardman MJ, et al. Estrogen
mediates acute elastic fibre homeostasis in skin. *bioRxiv.* 2019; 728865.
doi:10.1101/728865

Fig. 1 The effect of puerarin on the viability, injury and proliferative activity in high-passage NHDFs.

A, B: The effect of puerarin (pue) on cell viability of young (A) and senescent NHDFs (B) was analyzed by CCK-8 assay. n=5. C: The effect of cellular LDH release in high passage NHDFs. Data was obtained from LDH assay. n=6. D, E: The effect of puerarin on the cell proliferative activity in senescent NHDFs. Cells were stained for BrdU and nuclei by specific antibody and DAPI, respectively, and photographed at 100 × magnification (D). The percentage of BrdU positive cells per total cells was calculated from 15 microscopic fields for each group. (E). Scale bars = 100μm. *, p<0.05, **, p<0.01 vs control.

Fig. 2 The effect of puerarin on ROS generation in high-passage NHDFs.

ROS generation in senescent NFDFs (sene) with or without 50 μM puerarin (+pue 50) was measured by fluorescent ROS probe CellROX Green (A). CellROX intensity was calculated from 10 images for each group (B). Scale bars = 100μm. **, p<0.01 vs control.

Fig. 3 The effect of puerarin on cellular senescence in high-passage NHDFs.

A, B: The effect of puerarin on SA-β-galactosidase activity. Young and senescent (sene) NHDFs treated with 20 or 50 μM puerarin (pue) were stained by SA-β-galactosidase assay (A). The percentage of SA-β-galactosidase positive cells per total cells was calculated from 15 microscopic fields for each group. Scale bars = 100 μm. *, p<0.05, **, p<0.01 vs control.

Fig. 4 The effect of puerarin on the expression of SMA in high passage NHDFs.

A-B: Young or senescent (sene) NHDFs with or without 50 μM puerarin (+pue 50) were stained for SMA by the immunocytochemical method. The percentage of SMA positive cells per total cells was calculated from 15 microscope fields for each group. C The expression of SMA mRNA in

young and senescent NHDFs treated with or without 25-50 μ M puerarin (pue) for 72 h. Scale bars = 100 μ m. n=4 *; p<0.05, **; p<0.01 vs control.

Fig. 5 The effect of puerarin on gene expressions of papillary and reticular markers.

The expressions of PDPN (papillary marker), CNN1 (reticular marker) in young and senescent NHDFs treated with or without 25-50 μ M puerarin (pue) for 72 h. n=3-5 *; p<0.05, **; p<0.01 vs control.

Fig. 6 Estrogen receptor antagonist blocked puerarin-mediated reduction of SMA and CNN1.

The expressions of SMA and CNN1 mRNAs in senescent NHDFs treated with 50 μ M puerarin (+pue50) and estrogen receptor antagonist, 1 μ M fulvostrant (+Fulv) for 72 h. n=4 *; p<0.05, vs control, and \$; p<0.05 vs +pue50.

Table 1 Primers used for real-time PCR

Target	Forward	Reverse
SMA	ACTGAGCGTGGCTATTCCTTCGTT	GCAGTGGCCATCTCATTTTCA
PDPN	AAGAGCTGAAGGGTTACGCC	CACGGGTCATCTTCTCCCAC
CNN1	GGAAATTCGAGCCGGGGAA	TGGCAAACCTTGTGTTGCC
GAPDH	GTGGTCTCCTCTGACTTCAACA	GCTGTAGCCAAATTCGTTGTCA

(D)

(E)

(A)

Sene

+ pue 50

(B)

**CellRox intensity
(% of control)**

(A)**Sene****+pue25****+pue50** **(B)**

(A) **(B)** **(C)**

CNN1
(reticular marker)

PDPN
(papillary marker)