The decreased secretion of hyaluronan by older human fibroblasts under physiological conditions is mainly associated with the down-regulated expression of hyaluronan synthases but not with the expression levels of hyaluronidases

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Received: 17 December 2013 / Accepted: 18 February 2014 / Published online: 4 March 2014
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Abstract Although it has been reported that levels of hyaluronan are decreased in the dermis of aged skin, little is known about the cellular mechanism(s) underlying that hyaluronan deficiency. Since hyaluronan is produced by dermal fibroblasts and is secreted into the surrounding dermal tissues, we examined the secretion of hyaluronan by dermal fibroblasts and characterized its cellular mechanism using real-time RT-PCR and western blotting for its synthesizing and degrading enzymes, hyaluronan synthase and hyaluronidase, respectively. The secretion of hyaluronan by dermal fibroblasts derived from differently aged human donors, was higher in the younger human fibroblasts tested (0 and 19 years old) compared to the older human fibroblasts tested (39, 56 and 77 years old). The relative secretion levels of hyaluronan by the different human fibroblasts tested were attributable to the relative expression of hyaluronan synthases 1, 2, 3 but not hyaluronidases 1, 2 enzymes at the gene and protein

levels among those fibroblasts. These findings indicate that the deficiency of hyaluronan in the aged dermis might result from the down-regulation in the potential of older human fibroblasts to secrete hyaluronan and that decrease in secretory potential is mainly associated with the down-regulated expression of hyaluronan synthases, especially hyaluronan synthase 2, but not with the expression levels of hyaluronidases.

Keywords Human dermal fibroblasts · Hyaluronan · Hyaluronan synthase · Hyaluronidase · Ultraviolet B

Abbreviations

HA	Hyaluronan
HAS	Hyaluronan synthase
HYAL	Hyaluronidase
NHDFs	Normal human dermal fibroblasts
UVB	Ultraviolet B

Introduction

Hyaluronan (HA) is used to create an extracellular microenvironment that supports the typical physico-chemical and mechanical properties of the skin. HA thus serves important structural functions via mechanisms by which its high polymer length and polyanionic charge enables it to bind water, which in turn supports the volume expansion, turgidity and elasticity of the skin (Manuskiatti and Maibach 1996). In photo-

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aged skin, there is a marked deficiency of HA (Margelin et al. 1996; Takahashi et al. 1995, 1996) in addition to the fragmentation and loss of type I collagen fibrils due to the up-regulated activity of matrix-metalloproteinase (MMP)-1 (Fisher et al. 2008, 2009). Available evidence indicates that naturally aged, sun-protected skin and photoaged skin share important molecular features, including connective tissue damage, elevated matrix metalloproteinase levels and reduced collagen production (Varani et al. 2001, 2002). It has been reported that the content of HA significantly declines with increasing age in the dermis of sun-protected human skin (Longas et al. 1987), which seems to be associated with the decreased elasticity and the increased incidence of wrinkle formation in aged skin. Since those studies were carried out on the dermis of sun-protected skin, the age-dependent decrease in HA content could not be accounted for by the effects of UV, in which the dermal content of HA is markedly down-regulated by long-term exposure of the skin to UVB (Margelin et al. 1996; Takahashi et al. 1995, 1996; Dai et al. 2007). It is thus of considerable interest to determine the mechanism(s) by which HA levels in the dermis of sun-protected skin are down-regulated in an age-dependent manner.

HA is a linear polymer composed of repeating disaccharides (D-glucuronic acid; 1,3-*N*-acetylglucosamine; 1,4-*N*-acetylglucosamine) which is assembled from the respective activated nucleotide sugars (UDP-glucuronic acid, UDP-*N*-acetylglucosamine) at the inner plasma membrane by HA synthases (HAS). Three different HAS isoforms (HAS 1, 2 and 3) are known that reside in the plasma membrane and extrude the growing HA polymer into the extracellular space (Itano and Kimata 1996). Further, three different hyaluronidase (HYAL) isoforms (HYALs-1, 2 and 3) are known to cleave HA into limited fragments (Stern 2004) which in turn activate Toll-like receptors 2 and 4 and thereby modulate inflammatory responses (Termeer et al. 2002; Jiang et al. 2005) although the properties of HYAL3 are not well understood. Thus, the balance of HAS and HYAL activities is an essential factor in determining HA content in the dermis.

Since little is known about biological mechanisms that regulate the secretion levels of HA by normal human dermal fibroblasts (NHDFs) under non-stimulated physiological conditions, we examined the levels of HA secretion by NHDFs derived from donors of

different ages and used real-time RT-PCR and western blotting to elucidate the mechanism(s) underlying changes in HA secretion by comparing the secretion levels of HA with the expression of HASs and HYALs. Here we show that NHDFs derived from differently aged donors secrete HA in culture, with greater secretion of HA by NHDFs derived from younger (0 and 19 year old) donors compared with those derived from older (39 and 77 year old) donors. Our findings also indicate that the deficiency of HA in the aged dermis might result from the down-regulation in the potential of older human fibroblasts to secrete HA and that decrease in secretory potential under non-stimulated physiological conditions is mainly associated with the down-regulated expression of HAS, especially HAS2, but not with the expression levels of hyaluronidases.

Materials and methods

Materials

NHDFs derived from the dermis of sun-protected skin from donors of different ages were obtained from Kurabo (Tokyo, Japan). They include NHDFs from a <1 year old Caucasian male (0NHDF), a 19 year old Caucasian female (19NHDF), a 39 year old Japanese male (39NHDF-1), a 39 year old Caucasian female (39NHDF-2), a 43 year old Caucasian female (43NHDF), a 56 year old Japanese male (56NHDF) and a 77 year old Japanese female (77NHDF). Anti-human HAS1 (C-14:SC-23145), anti-human HAS2 (Y-14:SC34068), anti-human HAS3 (L-14:SC34202), anti-human HYAL1 (1D10:SC-101340), anti-human HYAL2 (M-19:SC49205) and anti- β -actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated goat polyclonal anti-mouse IgG was obtained from Transduction Laboratories (Franklin Lakes, NJ, USA). Other chemicals of reagent grade were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

NHDFs from differently aged donors were cultured in Dulbecco modified Eagle's medium (DMEM) supplemented with antibiotics/antimycotics, 2 mmol/L L-glutamine (PAA Laboratories GmbH, Cölbe, Germany) and

10 % fetal calf serum (FCS) in a CO₂ incubator (5 % CO₂) at 37 °C. In experiments to measure HA, genes and protein levels, NHDFs were cultured in DMEM without FCS.

Measurement of HA

NHDFs were seeded in DMEM with 10 % FCS and were cultured for 12 h. After changing the medium to fresh DMEM without FCS, HA secreted into the medium by NHDFs was measured at the indicated times of culture using an HA assay kit (Seikagaku Biobusiness Co., Ltd., Tokyo, Japan), which can detect HA with molecular weight ranging from 27 to 1,200 kDa, according to the instructions of the manufacturer (Kondo et al. 1991). Levels of HA are expressed as ng/ml/10⁶ cells.

Quantitative reverse transcription PCR

After changing the medium to fresh DMEM without FCS, levels of HAS1,2,3 and HYAL1,2 mRNAs in NHDFs were measured at the indicated times of culture using real-time RT-PCR as described previously by Suganuma et al. (2010). Primers with the following sequences were used: GAPDH: forward, 5'-GAAGGT-GAAGGTCGGAGTCAACG-3', reverse, 5'-AGTCC TTCCACGATAACCAAAGTTG-3' 384 bp; HAS1 (U59269): forward: 5'-CGCTAACTACGTCCCTCTG C-3', reverse: 5'-CCATGTTGACGCTGTACTGG-3'; HAS2 (U54804): forward: 5'-CCTCATCATCCAAA GCCTGT-3', reverse: 5'-AAACAGTTGCCCTTTGCA TC-3'; HAS3 (BC021853): forward: 5'-GTCAGTGGT CACG-GGTTTCT-3', reverse: 5'-ATTGGCCTCATTC CTGTGTC-3'; HYAL1 (U90094): forward: 5'-CAGA GCCGTGCCCTCTATC-3', reverse: 5'-ACTTTCTG CCCCTGGATGA-3'; HYAL2 (AJ000099): forward: 5'-CTCACGGGGCTTAGTGAGAT-30, reverse: 5'-GT GGTCAATGTGTCTCTGGG-3'.

Western blotting

After changing the medium to fresh DMEM without FCS, NHDFs were cultured for the indicated times of culture and were lysed in lysis buffer (50 mM Tris (pH 7.6), 150 mM NaCl and 1 % Triton X-100) plus protease inhibitors (Complete mini-tablets; Boehringer Mannheim, Mannheim, Germany) and were centrifuged at 13,000 rpm for 15 min. The supernatants were

harvested, lysed in two loading buffer (125 mM Tris (pH 6.8), 4.6 % sodium dodecyl sulfate (SDS), 20 % glycerol and 0.04 % pyronin Y), and then boiled for 5 min. Samples were solubilized in SDS sample buffer plus 50 mM dithiothreitol and boiled for 5 min. Total proteins from cultures of NHDFs were subjected to Western blotting with antibodies to HAS1/2/3, HYL1/2 or β -actin. Samples were separated on SDS-PAGE gels, transferred onto nitrocellulose membranes, blocked with 5 % nonfat dry milk in Tris-buffered saline (TBST) and probed with the above antibodies at room temperature for 1 h. Membranes were washed 3 times for 15 min with TBST, probed with peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ, USA or ICN Biomedicals Inc, Solon, OH, USA), then washed 3 times for 30 min in TBST and developed by ECL (Amersham). Blots were exposed to X-ray film for the specified times to detect bands; specific bands were quantitated by densitometric scanning and were analyzed with Quantity One® (Bio Rad).

Statistics

All data are expressed as mean $\pm$ SD ($n = 3$). For multiple comparisons, data were tested by one-way ANOVA, and subsequently using the Tukey multiple comparison test. p values <0.05 are considered statistically significant.

Results

Cell proliferation by NHDFs derived from donors of different ages

We examined the proliferation of NHDFs with different ages. Measurement of cell numbers revealed that while 0NHDFs and 43NHDFs showed an increase in cell number at 48 h of culture compared with 1 h of culture, 77NHDFs have a decreased number of cells at 48 h and an unchanged number of cells at 72 h of culture (Fig. 1).

Effects of subculture number on the secretion of HA

The level of HA secretion for 24 h of culture began to slightly decline after 20 subcultures and significantly

Fig. 1 Effects of aging on the proliferation of NHDFs. **A** 0NHDF, 13 subcultures, **B** 43NHDF, 14 subcultures, **C** 77NHDF 13 subcultures. NHDFs were seeded in DMEM with 10 % FCS and were cultured for 12 h. After changing the

medium to fresh DMEM without FCS, cell numbers of NHDFs were measured at 1, 48 and 72 h of culture. Results represent mean $\pm$ SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$ versus 1 h of culture

Fig. 2 Effects of subculture number on HA secretion for 24 h of culture. **A** 0NHDF, **B** 39NHDF, **C** 77NHDF. NHDFs were seeded in DMEM with 10 % FCS and were cultured for 12 h. After changing the medium to fresh DMEM without FCS, HA

secreted into the culture medium by NHDFs was measured at 24 h of culture using an HA assay kit. Results represent mean $\pm$ SD from five independent experiments. * $p < 0.05$, ** $p < 0.01$ versus 7 subcultures

Fig. 3 Time course and comparison of HA secretion by NHDFs with different ages. **A** 0NHDF: 13 subcultures, **B** 19NHDF: 4 subcultures, **C** 39NHDF: 14 subcultures, **D** 56NHDF: 17 subcultures, **E** 77NHDF: 13 subcultures. **F** Comparison of HA secretion levels at 72 h. Human fibroblasts were seeded in the DMEM with FCS and cultured for 12 h. After

exchanged with fresh DMEM without FCS, HA secreted into the culture medium of human fibroblasts was measured at indicated time of culture using an HA assay kit. Result represents mean $\pm$ SD from three independent experiments. **: $p < 0.01$ versus 0NHDF, #: $p < 0.01$ between 19 and 39 NHDFs

decreased at more than 25 subcultures in all NHDF lines tested (Fig. 2). Therefore, in subsequent studies to examine the secretion levels, we used NHDFs with <20 subcultures.

HA secretion by NHDFs with different ages

Among NHDF with ages of 0, 19, 39, 56 and 77 years old, the 0NHDF and 19NHDF showed a gradual increase in HA levels during 96 h of culture with a peak at 72 h and they had similar peak levels of HA

secretion at 72 h (Fig. 3). In contrast, 39NHDF exhibited similar but lesser gradual increases in HA levels compared to 0NHDF and 19NHDF during the 96 h of culture with a peak at 72 h (Fig. 3). 77NHDF showed the lowest level of HA secretion with a peak at 72 h (Fig. 3). Comparison of HA secretion levels revealed similar levels between 0NHDF and 19NHDF but there was a significant decrease in 39NHDF compared to 19NHDF, followed by a further slight decrease in 56NHDF and 77NHDF at a peak time of 72 h of culture (Fig. 3).

Fig. 4 The expression levels of genes encoding HASs (**A**) and HYALs (**B**) by NHDFs with different ages. **A: a** HAS1, **b** HAS2, **c** HAS3, **B: a** HYAL1, **b** HYAL2. 0NHDF: 13 subcultures, 19NHDF: 4 subcultures, 39NHDF: 14 subcultures, 77NHDF: 13 subcultures. NHDFs were cultured for 96 h in DMEM without FCS. mRNA levels at the indicated times of culture were

analyzed by real-time RT-PCR as described in the “[Materials and Methods](#)”. Results represent mean $\pm$ SD from three independent experiments. *: $p < 0.05$, **: $p < 0.01$ versus 0NHDF, #: $p < 0.05$, ##: $p < 0.01$ versus 19NHDF, ¶: $p < 0.05$, ¶¶: $p < 0.01$ versus 39NHDF

Expression levels of genes encoding HASs and HYALs by NHDFs with different ages

To associate the secretion levels of HA with the expression levels of HASs and HYALs by NHDFs with different ages, we selected 0NHDF, 19NHDF, 39NHDF and 77NHDF for the analysis of the gene and protein expression of HASs and HYALs. We picked those lines because the HA secretion levels by 0NHDF and 19 NHDF are higher than those by 39NHDF and 77NHDF. We examined the expression level of the genes encoding the HA synthesis enzymes, HAS 1, 2 and 3, and the HA degrading enzymes, HYAL 1 and 2, during 72 h of culture. While HAS1 mRNA transcript levels decreased in the order of 0NHDF = 19NHDF > 39NHDF >> 77NHDF at 1, 12, 24 and 60 h, it was at the highest level in 39NHDF at 36, 48 and 72 h of culture (Fig. 4A). Among NHDFs of different ages, 77NHDF had the lowest HAS1 mRNA transcript level during 72 h. The HAS2 mRNA transcript level decreased in the order of 0NHDF = 19NHDF > 39NHDF >> 77NHDF through 72 h of culture (Fig. 4A). Among NHDFs of different ages, 77NHDF had the lowest HAS2 mRNA transcript level during 72 h of culture. While the HAS3 mRNA transcript level decreased in the order of 0NHDF > 19NHDF > 39NHDF >> 77NHDF at 12, 24, 60 and 72 h of culture, 77NHDF had the lowest HAS3 mRNA transcript level during the 72 h of culture (Fig. 4A). Thus, comparison of the gene expression levels of HAS1, HAS2 and HAS3 indicate that the mRNA transcript levels of HAS have a tendency to decrease in the order of 0NHDF $\geq$ 19NHDF > 39NHDF > 77NHDF during the period of 72 h of culture, which is roughly paralleled by the HA secretion levels by the corresponding NHDFs.

While the HYAL1 mRNA transcript level decreased in the order of 39NHDF > 19NHDF > 77NHDF $\geq$ 0NHDF at 1, 36, 48 and 72 h of culture, it had the highest level in 19NHDF at 12, 24 and 60 h of culture (Fig. 4B). Among NHDFs of different ages, 0NHDF had the lowest HYAL1 mRNA transcript level at 1, 36, 48, 60 and 72 h of culture. While the HYAL2 mRNA transcript level decreased in the order of 0NHDF $\geq$ 19NHDF >> 39NHDF > 77NHDF at 12, 24, 48, 60 and 72 h of culture, 77NHDF had the lowest HYAL2 mRNA transcript level during 72 h of culture (Fig. 4B). Thus, comparison of the gene expression levels of HYAL1 and HYAL2 indicates that the mRNA transcript levels of HYALs have a tendency to decrease

in the order of 0NHDF $\geq$ 19NHDF > 39NHDF > 77NHDF during the period of 72 h of culture, which is roughly paralleled by the HA secretion levels by the corresponding NHDFs.

Expression levels of HAS and HYAL proteins by NHDFs with different ages

We next examined the protein expression levels of HAS 1, 2 and 3 and HYAL 1 and 2 during 96 h of culture. While the HAS1 protein level decreased in the order of 0NHDF > 39NHDF > 77NHDF at 1, 48 and 96 h of culture, it had the highest level in 0NHDF at 1, 48 and 72 h of culture (Fig. 5A). Among NHDFs of different ages, 77NHDF had the lowest HAS1 protein expression level during 96 h of culture. While the HAS2 protein level decreased in the order of 0NHDF $\geq$ 19NHDF > 39NHDF > 77NHDF at 1 and 48 h of culture, it had the highest level in 0NHDF at 1, 48 and 72 h of culture (Fig. 5A). Among NHDFs of different ages, 77NHDF had the lowest level of HAS2 protein during the 96 h of culture. While the level of HAS3 protein decreased in the order of 0NHDF > 39NHDF > 77NHDF at 1 h of culture, it had the highest and the lowest levels in 39NHDF and 77NHDF, respectively, at 72 and 96 h of culture (Fig. 5A). Thus, comparison of the protein levels of HAS1, HAS2 and HAS3 indicates that they have a tendency to decrease in the order of 0NHDF $\geq$ 19NHDF > 39NHDF > 77NHDF during 96 h of culture, which is roughly paralleled by the HA secretion levels by the corresponding NHDFs.

While the HYAL1 protein level decreased in the order of 0NHDF > 19NHDF > 39NHDF > 77NHDF at 48 and 72 h of culture, it had the highest level in 0NHDF at 1, 48 and 72 h of culture (Fig. 5B). Among NHDFs of different ages, 77NHDF had the lowest HYAL1 protein expression level at 48, 72 and 96 h of culture. While the level of HYAL2 protein decreased in the order of 0NHDF > 19NHDF > 39NHDF > 77NHDF during 96 h of culture, it had the highest level in 0NHDF during 96 h of culture (Fig. 5B). Among NHDFs of different ages, 77NHDF had the lowest HYAL2 protein expression level during the 96 h of culture although there is no significant difference in HYAL2 protein levels during 96 h of culture except between 0NHDF and 77NHDF at 72 and 96 h of culture. Thus, comparison of the protein levels of HYAL1 and HYAL2 indicates that they have a tendency to decrease

Fig. 5 The protein expression levels of HASs and HYALs by NHDFs with different ages. **A:** **a** HAS1, **b** HAS2, **c** HAS3, **B:** **a** HYAL1, **b** HYAL2. 0NHDF: 13 subcultures, 19NHDF: 4 subcultures, 39NHDF: 14 subcultures, 77NHDF: 13 subcultures. NHDFs were cultured for 96 h in DMEM without FCS.

Cell lysates obtained at the indicated times of culture were subjected to Western blotting analysis as described in the [Materials and methods](#). Results represent mean $\pm$ SD from 3 independent experiments. *: $p < 0.05$, **: $p < 0.01$ versus 0NHDF, #: $p < 0.05$ versus 39NHDF

in the order of 0NHDF > 19NHDF > 39NHDF > 77NHDF during the 96 h of culture, which is roughly paralleled by the HA secretion levels by the corresponding NHDFs.

Discussion

HA is one of the most abundant components of the dermis of human skin. In addition to supporting the volume expansion, turgidity and elasticity of the skin due to its potent water binding capacity, HA also plays an important role in cell migration and differentiation processes during wound healing and inflammation via its receptors that involve CD44 and RHAMM (receptor of HA-mediated motility) (Toole and Hascall 2002; Toole et al. 2002; Turley et al. 2002; Tammi et al. 2005). In relation to the age-dependent impairment in wound healing, aging fibroblasts have a defect in their phenotypic maturation that is required for tissue remodeling and the closure of wounds because of the dysfunction of HA-dependent CD44/epidermal growth factor receptor signaling. The cellular mechanisms of UVB-induced changes in HA in skin cells and tissues have been studied in detail (Südel et al. 2005; Averbek et al. 2006; Dai et al. 2007; Röck et al. 2011), and it has been shown that the loss of HA is closely associated with phenotypic characteristics of skin photo-aging (Margelin et al. 1996; Takahashi et al. 1995, 1996). In contrast, the effects of chronological aging on HA are much less well understood. It has been reported that the content of HA significantly declines with increasing age in the dermis of sun-protected human skin (Longas et al. 1987) and that several conditions that accelerate skin aging, such as estrogen deficiency, are associated with the loss of HA from the dermis (Kanda and Watanabe 2005). However, little is known about the cellular mechanism(s) responsible for the decrease of HA in the dermis during chronological aging because of the difficulty of culturing NHDFs from donors of different ages. Here we report the first comprehensive analysis of intrinsic aging-related production of HA by NHDFs derived from different chronologically aged donors. We found that the secretion of HA by NHDFs in culture occurs in a biphasic fashion with young fibroblasts secreting more HA than aged fibroblasts. This is consistent with a previous study by Simpson et al. (2009) which showed that young fibroblasts have

an increased baseline synthesis of HA compared with donor-matched aged fibroblasts, although 10–15 and 25–35 population doubling levels (PDLs) were used and are referred to as young and aged NHDFs, respectively. In our study, the lower levels of HA secretion by aged fibroblasts does not seem to occur as a consequence of the senescence of NHDFs because fibroblasts were used at <20 subcultures and thus had not declined in their HA secretion potential. Since much larger numbers of NHDFs derived from donors of different ages are required to confirm these findings, we can only speculate that the decrease in HA content in the dermis of sun-protected skin with age is attributable to the decreased ability of aged NHDFs to secrete HA compared with younger NHDFs.

Since it is likely that HA secretion levels occur as a consequence of the balance between HA production and degradation, we compared the expression levels of HAS1, HAS2 and HAS3 and HYAL1 and HYAL2 in NHDFs derived from differently aged donors. Comparison of HAS1, HAS2 and HAS3 mRNA levels revealed that HAS2 gene expression levels are higher than HAS1 and HAS3, whereas HYAL2 gene expression predominates over HYAL1, which is consistent with a similar study by Averbek et al. (2006). In comparison of HAS mRNA levels among NHDFs derived from differently aged donors, while fibroblasts from the oldest donor (77NHDF) have the lowest levels of HAS1, HAS2 and HAS3 mRNAs at all culture time points, two kinds of NHDFs from younger donors (0NHDF and 19NHDF) have the highest mRNA levels of HAS1, HAS2 and HAS3 at all culture time points with mRNA levels similar to each other. This indicates that HAS mRNA levels, especially HAS2, are paralleled by the secretion levels of HA in different NHDFs, which suggests that HAS2 plays a key role in positively regulating the secretion levels of HA.

In comparison of HAS protein levels between NHDFs derived from different age donors, while fibroblasts from the oldest donor (77NHDF) have the lowest levels of HAS1, HAS2 and HAS3 proteins at all culture time points, two kinds of fibroblasts from younger donors (0NHDF and 19NHDF) have the highest levels of HAS1, HAS2 and HAS3 proteins at all culture time points with protein levels similar to each other. This indicates that, consistent with the trend for the expression levels of HAS genes, HAS protein levels, especially HAS1 and HAS2, are

paralleled by the secretion levels of HA in different NHDFs, which also suggests that HAS2 plays a key role in positively regulating the secretion level of HA. Taken together, these results for HAS expression seem to indicate that the expression of HAS, especially HAS2, at both the gene and protein levels, mainly reflects the secretion levels of HA. These results are consistent with another study using UVB-exposed skin fibroblasts, which demonstrated that HAS2 is the predominant isoform expressed in skin fibroblasts and the extent of HAS2 down-regulation correlated with the UVB-induced decrease of HA secretion (Röck et al. 2011).

In comparison of HYAL mRNA levels among NHDFs derived from differently aged donors, while fibroblasts from the oldest donor (77NHDF) have the lowest mRNA levels of HYAL1 and HYAL2 at all culture time points, two kinds of fibroblasts from younger donors (0NHDF and 19NHDF) have higher levels of HYAL2 mRNA at all culture time points with mRNA levels similar to each other. This indicates that HYAL mRNA levels, especially HYAL2, are roughly paralleled by the secretion levels of HA in individual NHDFs. In comparison of HYAL proteins among NHDFs derived from differently aged donors, while fibroblasts from the oldest donor (77NHDF) have the lowest levels of HYAL1 and HYAL2 proteins at all culture time points, fibroblasts from the youngest donor (0NHDF) have the highest levels of HYAL1 and HYAL2 proteins at all culture time points. This indicates that, consistent with the trend for HYAL genes, the two HYAL protein levels are roughly paralleled by the secretion levels of HA in individual NHDFs. Upon comparison with changes in HA secretion by individual NHDFs, these results for HYAL expression seem to indicate that the expression of HYALs at both the gene and protein levels are not associated with the decrease in HA secretion by individual NHDFs. As summarized in Table 1, the sum of these findings suggests that higher or lower levels of HA secretion by individual NHDFs is not mainly due to changes in HYAL expression, but rather to the up- or down-regulation of HAS expression, especially HAS2.

HAS2 is a major HAS expressed by NHDFs and produces high molecular mass HA ($2\text{--}4 \times 10^6$ Da), while HAS3 is a keratinocyte-specific HAS which synthesizes smaller HA ($0.4\text{--}2.5 \times 10^5$ Da) (Itano and Kimata 1996). As for the mechanism(s) underlying

Table 1 Summary on the levels of HA secretion and the expression of HAS/HYAL at gene and protein levels between younger and older NHDFs

	Expression level	Younger NHDFs	Older NHDFs
Hyaluronan secretion	ng/ml/ 10^6 cells	higher	lower
Hyaluronan synthase 1	gene	higher	lower
	protein	higher	lower
Hyaluronan synthase 2	gene	higher	lower
	protein	higher	lower
Hyaluronan synthase 3	gene	higher	lower
	protein	higher	lower
Hyaluronidase 1	gene	higher	lower
	protein	higher	lower
Hyaluronidase 2	gene	higher	lower
	protein	higher	lower

the age-related down-regulation of HAS expression, there are several possibilities in relation to aging changes in fibroblasts. Since levels of HAS mRNAs continue to increase after culture in serum-free medium, one may speculate that fibroblasts in culture secrete cytokines which stimulate HA secretion. Thus, it is probable that aged fibroblasts produce down-regulated levels of cytokines that are capable of stimulating HA synthesis. It has been reported that transforming growth factor β (TGF- β) up-regulates the expression of both the HAS1 and HAS2 genes in human fibroblasts (Sugiyama et al. 1998). Further, the cytokines TGF- β , platelet-derived growth factor (PDGF) (Heldin et al. 1989; Suzuki et al. 1995), interferon γ (IFN- γ), tumor necrosis factor α (TNF- α) (Elias et al. 1988), interleukin-1 (IL-1) (Postlethwaite et al. 1989) and epidermal growth factor (EGF) (Hata et al. 1988) have been shown to stimulate HA synthesis by fibroblasts. Because there are no available reports on possible age-dependent changes in cytokine secretion by NHDFs, it would be of interest to determine the secretion levels of those cytokines in NHDFs derived from donors of different ages.

As another possibility involved in the down-regulated expression of HAS2 in aged NHDFs, it should be noted that collagen fragments inhibit HA synthesis and HAS2 expression (Röck et al. 2011). It is well known that a hallmark of skin aging is the cleavage of collagen by matrix metalloproteinases (MMP) (Fisher et al. 1996). MMP-1 levels are also elevated in aged human skin (Fisher et al. 2009). Available evidence

supports the possibility that aged NHDFs have a higher level of MMP-1 expression which results in an increased content of fragmented collagen compared with young NHDFs. Since collagen fragmentation promotes oxidative stress and elevates MMP-1 levels in fibroblasts (Fisher et al. 2009), such a positive feedback loop coupling age-dependent MMP-catalyzed collagen fragmentation (CF) and CF-induced down-regulation of HAS may facilitate the deficiency of HA in aged NHDFs.

In conclusion, our findings indicate that the deficiency of HA in the aged dermis might result from the down-regulation in the potential of older human fibroblasts to secrete HA and that decrease in secretory potential under non-stimulated physiological conditions is mainly associated with the down-regulated expression of HAS, especially HAS2, but not with the expression levels of hyaluronidases.

Conflict of interest The authors have declared that no conflict of interest exists.

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