Development of human skin equivalents mimicking skin aging : contrast between papillary and reticular fibroblasts as a lead

Janson, D.

Chapter 3

Effects of serially passaged fibroblasts on dermal and epidermal morphogenesis in human skin equivalents

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Abstract

Serial passaging has a profound effect on primary cells. Since serially passaged cells show signs of cellular aging, serial passaging is used as an *in vitro* model of aging. To relate the effect of *in vitro* aging more to *in vivo* aging, we generated human skin equivalents. We investigated if human skin equivalents generated with late passage fibroblasts show characteristics of aged skin when compared with human skin equivalents generated with early passage fibroblasts.

Late passage fibroblasts had enlarged cell bodies and were more often positive for myofibroblast marker α-smooth muscle actin, senescence associated β-galactosidase and p16 compared with early passage fibroblasts. Skin equivalents generated with late passage fibroblasts had a thinner dermis, which could partly be explained by increased matrix metalloproteinase-1 secretion. In equivalents generated with late passage fibroblasts epidermal expression of keratin 6 was increased, and of keratin 10 slightly decreased. However, epidermal proliferation, epidermal thickness and basement membrane formation were not affected.

In conclusion, compared with human skin equivalents generated with early passage fibroblasts, human skin equivalents generated with late passage fibroblasts showed changes in the dermis, but no or minimal changes in the basement membrane and the epidermis.

Introduction

Like any organ in the human body, skin is affected by aging. Aged skin is characterized by a flat epidermis and loss of dermal matrix (1-3). On macroscopic level this leads to, amongst others, wrinkle formation, discoloration (aging spots) and telangiectasia (4, 5). Functionally, aged skin is less capable of wound healing and loses barrier function (dry skin) (6).

Skin aging is typically divided in two categories: intrinsic aging and photoaging. Intrinsic aging is caused by the aging processes underlying "general" organismal aging, while photoaging is caused by UV damage, and is only found in the body parts that are sun exposed (7). Intrinsically aged and photoaged skin show different aging characteristics, both macroscopically and microscopically. For example, photoaged skin is characterized by epidermal hyperplasia and increased production and breakdown of matrix molecules, while intrinsically aged skin is characterized by a general atrophy in many skin components (8). The focus of this study is on intrinsic aging.

Human primary cells have a limited proliferative lifespan *in vitro* (9). A large number of studies have been conducted in which prolonged serial passaging, usually of fibroblasts, was used as a model for aging (10-14). These studies are performed mostly in monolayer cell populations. To relate the effect of *in vitro* aging more to *in vivo* aging, we generated human skin equivalents (HSEs) with late passage fibroblasts and investigated to what extent these HSEs resembled aged skin when compared with HSEs generated with early passage fibroblasts.

The use of HSEs allows for a more direct relation between prolonged serial passaging of fibroblasts and (skin) aging by incorporating the fibroblasts in a microenvironment more representative of their original tissue. In addition, HSEs allow not only the study of the fibroblasts themselves, but also their interactions with keratinocytes, basement membrane formation and their effect on the extracellular matrix generation. HSEs are a dynamic tool for research in skin aging. For example, they can be used to study damage caused by photoaging or to test the efficacy (and toxicity) of anti-aging compounds (15-18).

Several characteristics related to skin aging *in vivo* can be studied in HSEs. These include damage and reduced formation of the basement membrane (19, 20), decreased fibroblast matrix production and increased matrix degradation (3, 21), fibroblasts senescence (22-24), decreased epidermal proliferation (25), increased expression of epidermal activation related keratins (26) and decreased expression of epidermal differentiation markers (27). One of the most prominent histological characteristics of aged skin, the disappearance of the rete ridges (1), can not be investigated in HSEs, since rete ridges are not formed *in vitro*.

We hypothesized that HSEs generated with late passage fibroblasts show characteristics of aged skin when compared with HSEs generated with early passage fibroblasts. Early passage fibroblasts then represent a young population, while late passage fibroblasts represent an aged population (10, 28-30). The presented results show that several, but not all characteristics of aged skin can be mimicked in HSEs generated with late passage fibroblasts.

Materials and Methods

Cell culture

Human skin cells were isolated from surplus skin from cosmetic surgery (abdominal or mammary tissue). Fibroblasts from six middle-aged female donors were used for experiments on monolayer. Three of these were used for generation of human skin equivalents, aged 44, 56 and 60 years.

For fibroblast isolation, first, epidermis was separated from the dermis by overnight incubation of the skin with dispase II (Roche Diagnostics, Almere, The Netherlands). Fibroblasts were isolated from the dermis by incubation with a solution consisting of collagenase II (Invitrogen, Breda, The Netherlands) and dispase II (ratio 3:1) at 37°C for 2 hours. The cells were filtered using a 70 μm cell strainer, and cultured in fibroblast medium at 37°C and 5 % CO2 until subconfluency. Fibroblast medium consisted of Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 5 % Fetal Bovine Serum (FBS, HyClone/Greiner, Nürtingen, Germany), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen). Part of the fibroblasts was frozen in liquid nitrogen (early passage controls) and part was kept in culture for 30 passages (1:3 subcultures) to obtain late passage populations. A growth curve of late- versus early passage fibroblasts was generated by seeding 5000 fibroblasts into 6-wells plates. Cells were counted with a Bürker counting chamber after 4, 6, 8 and 11 days.

Keratinocytes were isolated from the epidermis through incubation with trypsin at 37°C for 15 minutes. After trypsin inactivation, the cells were filtered using a 70 μm cell strainer (BD Biosciences, Breda, The Netherlands) and cultured in keratinocyte medium at 37°C and 7.3% CO2 until subconfluency. Keratinocyte medium consisted of 3 parts DMEM and 1 part Ham's F12 medium (Invitrogen) supplemented with 5% FBS, 0.5 μM hydrocortisone, 1 μM isoproterenol, 0.1 μM insulin (Sigma-Aldrich, Zwijndrecht, The Netherlands), 100 U/mL penicillin and 100 μg/mL streptomycin.

Fibroblast Derived Matrix

For detailed procedure see (31). In brief, fibroblast-derived matrices (FDMs) were generated by seeding 200.000 fibroblasts into a polyester permeable support (6 well plates with 0.4 μm pore size Transwell inserts, Corning Incorporated, Schiphol-Rijk, The Netherlands). FDMs were cultured for three weeks in CNT-05 (CELLnTEC, Huissen, The Netherlands) medium supplemented with 50 μM ascorbic acid phosphate (Sigma-Aldrich). Culture medium was refreshed twice a week.

Human skin equivalents

Full-thickness human skin equivalents (HSEs) were generated by seeding 500.000 primary keratinocytes (pool of two different donors) in early passage onto the FDMs. Cultures were incubated overnight in keratinocyte medium as described above. After two days medium was changed to keratinocyte medium as above, but with 1% FBS and supplemented with 53 μM selenious acid, 10mM L-serine, 10 μM L-carnitine, 1 μM dL-α-tocopherol-acetate, 250 μM ascorbic acid phosphate, 24 μM bovine serum albumin and a lipid supplement containing 25 μM palmitic acid, 15 μM linoleic acid and 7 μM arachidonic acid (Sigma-Aldrich). After another two days the cultures were exposed to the air. From then, HSEs were cultured in supplemented keratinocyte medium as described above, except that FBS was omitted and the concentration of linoleic acid was increased to 30 μM. Medium was refreshed twice a week. After two weeks of air-exposed culture, the HSEs were processed for analysis.

Morphological and immunohistochemical analysis

HSEs were harvested in two parts, one half was snap-frozen in liquid nitrogen while the other half was fixed in 4% formaldehyde, dehydrated and paraffin embedded. Global morphological analysis was performed on 5 μm formalin-fixed paraffin-embedded through staining with haematoxylin and eosin (HE). Immunohistochemical analysis was either performed on paraffin embedded material or cryosections. For the paraffin material, 5 μm sections were cut and rehydrated in xylene and ethanol. For cryosections, 5 μm sections were cut and fixed with acetone. For immunocytochemical analyses of monolayer cultures, fibroblasts were grown on a glass slide. These were washed twice with PBS and fixed for 10 min with 4% formaldehyde. Following incubation with the primary antibody, sections were stained with avidin-biotin-peroxidase system (GE Healthcare), as described by manufacturer's instructions. Staining was visualized with AEC and sections were counterstained with haematoxylin. Fluorescent stainings were visualized by using secondary antibodies conjugated to Cy3 and counterstained with DAPI.

To quantify the fraction of proliferating keratinocytes, the number of Ki67 positive basal keratinocytes was divided by the total number of basal keratinocytes.

Antibodies

The antibodies used in this study were: α-smooth muscle actin (1A4, Sigma-Aldrich), Keratin 6 (KS6.KA12, Sanbio – Monosan, Uden, The Netherlands), Keratin 10 (DE-K10, Abcam, Cambridge, UK), Collagen type I (Southern Biotech, Birmingham, AL, USA), Collagen type III (Southern Biotech), Collagen type IV (PHM12, Chemicon, Amsterdam, The Netherlands), Laminin 332 (BM165, kind gift from Dr. A. Aumailly) and Ki67 (MIB1, DAKO).

Beta galactosidase staining

Cells and frozen tissue sections were analysed for beta galactosidase staining at pH 6. Frozen tissue sections were thawed in 4% formaldehyde (+/- 40 min). Cells grown in monolayer were washed twice with PBS and fixed in 4% formaldehyde for 10 min. For both, this was followed by 3 washes with wash solution (100 mM Na2HPO4 set to pH 6 with 100 mM Citric Acid). Staining was performed O/N at 37°C in freshly made staining solution (5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 150 mM NaCl, 40 mM Na_2HPO_4, 1 mg/mL bromo-chloro-indolyl-galactopyranoside (X-Gal), set to pH 6 with 100 mM Citric Acid). After the incubation tissue sections were washed 3 times with wash solution and post-fixed with 4% formaldehyde overnight. Sections were then dehydrated with ethanol and isopropanol, and embedded in paraffin. Sections were cut, rehydrated with isopropanol and ethanol and counterstained with haematoxylin. For monolayer slides the post-fixing was 10 min in 4% formaldehyde. This was followed by counterstaining with haematoxylin.

p16 analyses

Immunolabeling of p16 was performed on paraffin embedded material with the CinTec p16 kit (mtm laboratories, USA MA), according to manufacturer's instructions. The percentage of p16 positive cells was determined by counting and dividing the p16 positive fibroblasts and the total number of fibroblasts in at least 3 fields per section.

MMP-1 ELISA

Quantification of matrix metalloproteinase-1 (MMP-1) was performed with the Quantikine MMP-1 kit (R&D Systems). Culture medium of the HSEs was collected during

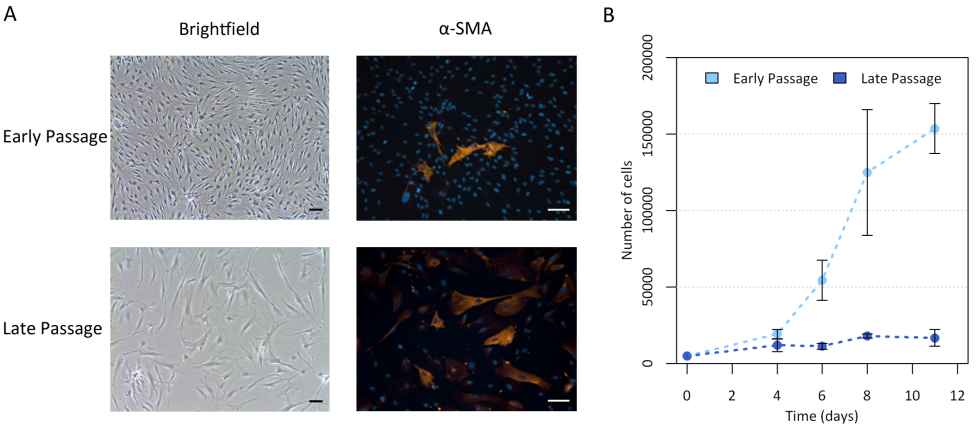

Figure 1: Monolayer characteristics of early – and late passage fibroblasts (2 and 30 passages respectively). A) Early passage fibroblasts are smaller and reach higher culture densities compared with late passage fibroblasts. Late passage fibroblasts are more often positive for myofibroblast marker α-SMA. Scale bar: 100 μm. B) Late passage fibroblasts divide very slowly, but still have replicative capability left. The data represent the average of three different donors. Error bars depict SEM.

refreshment throughout the entire culture period. Measurements and data analysis were performed according to the manufacturer's protocol.

Results

Fibroblast morphology and proliferation after serial passaging

After prolonged culture fibroblasts developed characteristics as described earlier (9, 28). While early passage fibroblasts formed dense cultures and had small cell bodies, late passage fibroblasts were enlarged and showed increased expression of myofibroblast marker α-smooth muscle actin (α-SMA, figure 1A). Another important feature of passaging is the loss of replicative capacity. The late passage fibroblasts showed a significantly lower growth speed (figure 1B). However, they still showed some replicative ability.

Expression of β-gal and p16 in late passage cells

During serial passaging, some cells will enter a non-dividing, senescent state. To determine the extent of senescence in the cultures, we stained for two markers: tumor suppressor p16 and beta-galactosidase staining at pH6 (β-gal). Both have been used before as a marker of senescent fibroblasts in monolayer and in *in vivo* skin (22-24, 32).

Both markers were more expressed in late passage cultures than in early passage cultures (figure 2A). However, p16 was expressed in more fibroblasts than β-gal (35 % vs. 25 %) (figure 2B).

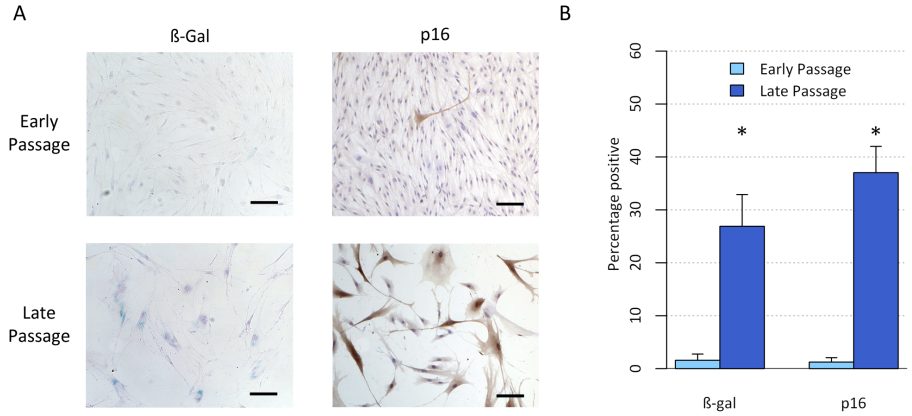

Figure 2: Immunohistochemical analysis of senescence markers p16 and β-gal in monolayer cultures of early- and late passage fibroblasts (2 and 30 passages respectively). A) Staining of either β-gal or p16. Late passage fibroblasts showed significant staining for the senescence markers, while in early passage fibroblasts expression was minimal. Scale bars: 100 μm. B) Quantification of β-gal and p16 staining. The percentage of positive cells was higher with p16 than with β-gal, but this difference was not significant. The data represents the average of three different donors. Error bars depict SEM. * = $P < 0.05$, early- compared with late passage, t-test.

Late passage fibroblasts show a decreased fibroblast derived matrix

HSEs were generated with early- and late passage fibroblasts. Three donors were used that had approximately 15 – 20 % β-gal positive fibroblasts in late passage populations and <1 % β-gal positive fibroblasts in the early passage populations in monolayer culture. FDM dermal equivalents were generated with 200.000 fibroblasts and seeded with keratinocytes after three weeks of culture. Passaging had a profound effect on the matrix deposition of the fibroblasts; HSEs generated with late passage fibroblasts had a thinner dermis (50 – 150 μm) than HSEs generated with early passage fibroblasts (200 – 400 μm) (figure 3). Collagen type I and III were expressed abundantly throughout the dermis. The amount and ratio of expression were not different between HSEs generated with early- and late passage fibroblasts (data not shown).

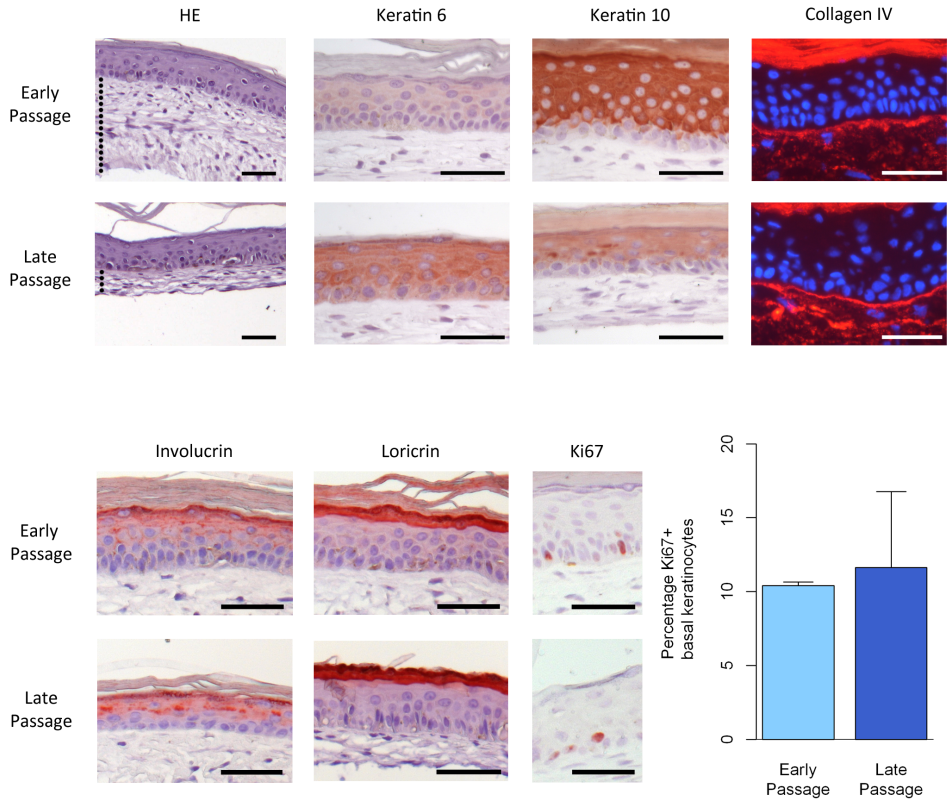

Figure 3: Immunohistochemical analysis of HSEs generated with early- and late passage fibroblasts. Representative pictures of a single fibroblast donor (out of three) are shown. Morphological analysis by HE staining showed that late passage fibroblasts generated a thinner dermis than early passage fibroblasts (marked by dotted line). Epidermal activation associated marker keratin 6 was higher expressed in late passage HSEs. Keratin 10 was expressed suprabasally in both low- and late passage equivalents, but the intensity was decreased in late passage HSEs. Collagen type IV was expressed predominantly in the basement membrane, but not differently in late passage HSEs. Terminal differentiation markers involucrin and loricrin were not differently expressed between early- and late passage fibroblasts. Epidermal proliferation was not different, as measured by counting Ki67-positive basal keratinocytes. Scale bars: 50 μm.

MMP1 secretion is increased in late passage HSEs

To further investigate the effect of the late passage fibroblasts on dermal formation, we examined the secretion of MMP1 in the culture medium using ELISA. Culture supernatants were collected during the formation of the dermal equivalent, before seeding of keratinocytes. In two out of three HSEs generated with late passage fibroblasts the secretion of MMP1 was significantly increased compared with their early passage counterparts of the same donor (figure 4).

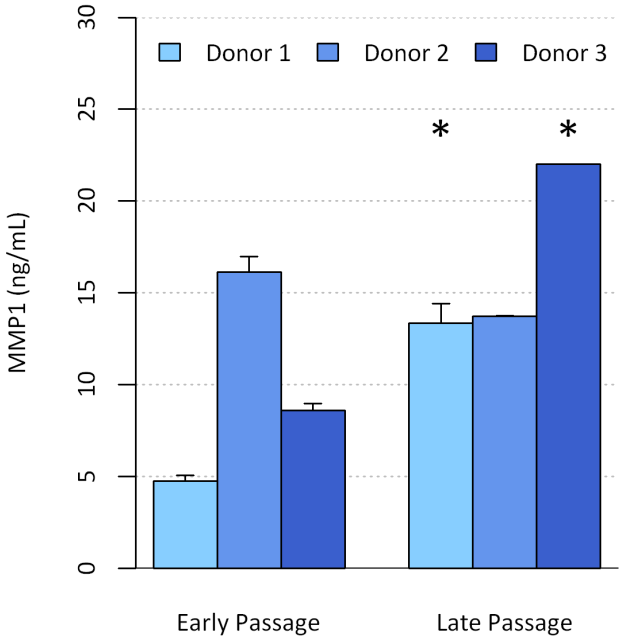

Figure 4: MMP1 concentrations in supernatants of HSEs before keratinocytes were seeded. Two out of three late passage populations had increased MMP1 expression compared with early passage fibroblasts of the same donor. Error bars depict the SD of technical replicates. * = $P < 0.05$, t-test.

Effects of late passage fibroblasts on epidermis of human skin equivalents

Next, the effect of late passage fibroblasts on the epidermal morphogenesis was investigated. All HSEs showed a nicely formed epidermis (+/- 5-6 viable cell layers) consisting of a stratum basale, stratum spinosum, stratum granulosum and a stratum corneum (figure 3). Immunohistochemical analyses were performed for epidermal activation associated

keratin 6, early-differentiation marker keratin 10, basement membrane markers collagen type IV and laminin 332, and cell proliferation marker Ki67.

Keratin 6 showed a weak expression in HSEs generated with early passage fibroblasts, but was abundantly expressed in the suprabasal layers of HSEs generated with late passage fibroblasts. Keratin 10 was expressed in the suprabasal layers of the epidermis in both conditions, but the intensity was lower in late passage HSEs. Collagen type IV and laminin 332 (not shown) were expressed similarly at the basement membrane, irrespective of fibroblast passage (figure 3). The percentage of proliferating basal keratinocytes was not different between both conditions, as determined by Ki67 staining; the percentage of proliferating basal keratinocytes was 10 % in HSEs generated with early passage fibroblasts and 11 % in HSEs generated with late passage fibroblasts (average of three donors)(figure 3).

Expression β-gal and p16 in HSEs

It was investigated whether the expression of β-gal and p16 in HSEs were similar to monolayer cultures. The HSEs were generated with late passage fibroblast populations that contained approximately 15 – 20 % β-gal positive fibroblasts or with early passage populations containing <1 % β-gal positive fibroblasts in monolayer culture. However, when the HSEs were harvested after 14 days of air-exposed culture, virtually all fibroblasts stained positive for β-gal, regardless of the fibroblast populations used (data not shown). Keratinocytes were not positive for β-gal in these HSEs.

In contrast, p16 expression was significantly increased in fibroblasts of HSEs generated with late passage populations compared with early passage controls (figure 5). There was considerable variability among the donors. In addition, there was no correlation between the number of p16-positive fibroblasts measured after harvesting and the morphology of the HSEs. Occasionally, a few keratinocytes were positive for p16, both in HSEs were generated with early- and late passage fibroblasts.

Discussion

We hypothesized that HSEs generated with late passage fibroblasts would show characteristics of aged skin. Several characteristics of *in vivo* aged skin are present in HSEs generated with late passage fibroblasts, such as decreased dermal thickness and increased expression of epidermal activation associated marker (keratin 6) (3, 26). However, several characteristics are also lacking, such as changes in epidermal proliferation and in the basement membrane (20, 25). The findings are summarized in Table 1.

Some characteristics of aged skin might only be visible when a higher percentage of senescent cells is present; in this study we generated HSEs with populations that had

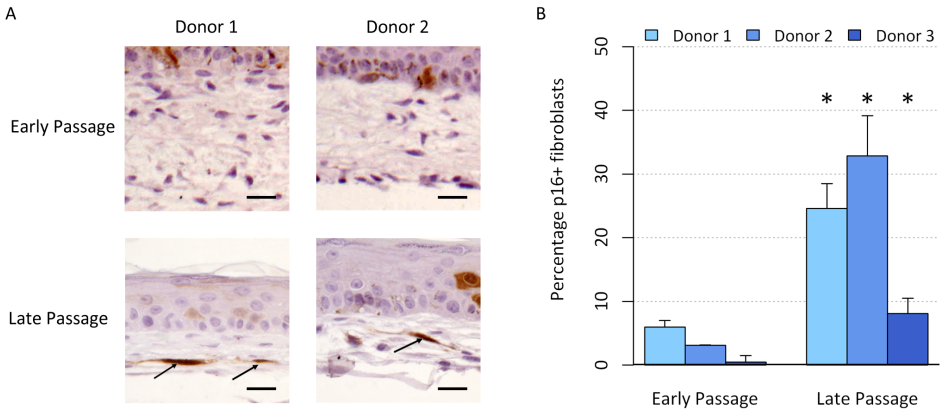

Figure 5: Immunohistochemical analysis of p16 expression in HSEs generated with early- and late passage fibroblasts. A) Pictures of HSEs stained with p16. There were more fibroblasts positive in the HSEs generated with late passage fibroblasts than in early passage controls (arrows). Occasionally, keratinocytes were positive for p16 as well. Scale bars: 20 µm. B) Quantification of the number of p16 positive fibroblasts in three donors. The percentage of p16 positive fibroblasts was significantly increased in HSEs generated with late passage populations. Error bars depict the SD of the counting. * = $P < 0.05$, t-test.

about 15 – 20 % β-gal positive cells in monolayer. In our experience, HSEs generated with fibroblasts that have higher percentages of β-gal positive cells (> 30 %) do not generate viable HSEs (data not shown). The extracellular matrix created by these fibroblasts (or, more precisely, lack thereof) is not able to support epidermal proliferation and differentiation and can therefore not function as a dermal equivalent. As a consequence these matrices will not form a proper, full-thickness HSEs.

The lack of changes in keratinocyte proliferation and basement membrane formation were unexpected. The basement membrane depends on deposition of matrix both from the fibroblasts as well as the keratinocytes (33). Furthermore, after the cultures are air-exposed no serum is added to the medium. As such, the keratinocytes depend on fibroblasts for stimulation with growth factors. It is possible that there are still enough "normal" functioning fibroblasts to support basement membrane formation and epidermal proliferation. Or the serially passaged fibroblasts still are capable of stimulating keratinocyte proliferation and basement membrane formation.

As mentioned before, when fibroblast populations with a high percentage of β-gal cells (> 30%) were used, no matrix is formed. This severely disturbs epidermal morphogenesis after keratinocytes are seeded. This stresses the importance of the matrix itself for the growth of keratinocytes; the HSEs with late passage fibroblasts have reduced matrix

Skin characteristic	Change in aged skin *in vivo*	Change in late passage HSE *in vitro*
Fibroblast senescence	Increased	Increased
Expression of epidermal activation associated markers (keratin 6)	Increased	Increased
Basement membrane (collagen type IV and laminin 332)	Decreased	No change detected
Epidermal proliferation	Decreased	No change detected
Matrix deposition	Decreased	Decreased
MMP1 production	Increased	Partly, increased in 2/3 donors
Rete ridges	Lost	No rete ridges present
Keratin 10 expression	Decreased	Decreased

Table 1: Summary of the investigated characteristics of aged skin and if they are affected in HSEs generated with late passage fibroblasts.

formation, but there is still matrix present for the keratinocytes to grow on. Furthermore, an important aspect of skin aging is damage to the extracellular matrix. The matrix contains several components with a long half-life. For example, the half-life of collagen type I in the skin is approximately 15 years (34). As such, the matrix is sensitive to accumulation and retention of damage. The matrix secreted by late passage fibroblasts in HSEs is obviously not affected by years of accumulated damage. The next question then is whether the matrix secreted by late passage fibroblasts is different in composition from the matrix secreted by early passage fibroblasts, or whether there is only reduced, but otherwise normal, matrix secretion. The data presented above hints at the latter, but the composition and structure of the matrix have not been investigated in detail.

There is a caveat to the investigation of senescence in HSEs: the markers. β-gal is not a suitable marker to detect senescent fibroblasts in HSEs. In proliferative, monolayer cultures β-gal can accumulate specifically in the non-dividing, likely senescent cells and β-gal can be used as a proxy for senescence (35). However, β-gal is not a senescence marker in high density, non-proliferating cultures; it will accumulate in all cells regardless of senescence (36). In several types of HSEs, and especially in the type used in this study (fibroblast derived matrix), fibroblasts are seeded at high density and do not show any proliferation after seeding. Therefore, β-gal staining will become positive in virtually all fibroblasts in HSEs, and not in the proliferating keratinocytes. P16 was expressed more among expected lines in HSEs generated with late passage fibroblasts. However, there is discrepancy between β-gal and p16 staining in monolayers. And p16 can be induced by

other stimuli than senescence as well, such as UV (37).

In conclusion, compared with HSEs generated with early passage fibroblasts, HSEs generated with late passage fibroblasts showed changes in the dermis, but no or minimal changes in the basement membrane and the epidermis.

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