Different Gene Expression Patterns in Human Papillary and Reticular Fibroblasts

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The dermis contains two distinct layers: the papillary and the reticular layers. *In vitro* cultures of the fibroblasts from these layers show that they are different. However, no molecular markers to differentiate between the two subtypes of fibroblasts are known. We performed gene expression analysis on cultured fibroblasts isolated from the papillary and reticular dermis. In all, 116 genes were found to be expressed differentially. Of these, 13 were validated by quantitative reverse transcriptase–PCR analysis and two markers could be validated at the protein level in monolayer cultures. Three markers showed differential expression in *in vivo* skin sections. The identified, characteristic markers of the two fibroblast subpopulations provide useful tools to perform functional studies on reticular and papillary fibroblasts.

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INTRODUCTION

Originally believed to be a relatively uninteresting cell population, fibroblasts have become a main focus of research interest. On one hand, this is based on the easy isolation and culture techniques required to obtain fibroblasts from human skin. On the other hand, because fibroblasts have shown to be a very dynamic cell population involved in many physiological processes, definitely not restricted to matrix homeostasis. These include communication with vasculature, nervous system and epithelial tissues, the immune response, and wound healing (Sorrell and Caplan, 2009). Furthermore, fibroblasts are known to be involved in several disease-related processes, for example, cancer invasion (Kalluri and Zeisberg, 2006). Another important role of fibroblasts is their implication in the aging process. Because skin fibroblasts are easily accessible, a lot of aging research is performed on monolayer fibroblast cultures (e.g. Maier and Westendorp, 2009).

Fibroblasts from different locations and tissues are distinct (Chang *et al.*, 2002; Rinn *et al.*, 2006, 2008). This is also true for their location within the skin where they populate the two dermal layers, the superficial papillary dermis (300–400 μm) and the underlying reticular dermis. The papillary dermis is characterized by a relatively thin extracellular matrix and a high cell density, whereas the reticular dermis has a very dense network of matrix fibers and a low cell density (Ross and Pawlina, 2011). The constituents of the matrix are also distinct in the two layers, in particular decorin is found primarily in the papillary dermis (Schonherr *et al.*, 1993). Other matrix constituents that differ between the papillary and reticular dermis include, but are not limited to: versican, collagen IV, collagen XII, and collagen XVI (Sorrell and Caplan, 2004).

When fibroblasts of the respective layers are cultured, they preserve distinct morphological characteristics. Reticular fibroblasts have a squarer and stretched appearance, while papillary fibroblasts generally have a lean, spindle-shaped morphology. Furthermore, differences were found in proliferation (Harper and Grove, 1979; Azzarone and Macieira-Coelho, 1982), matrix production in culture (Tajima and Pinnell, 1981; Schonherr *et al.*, 1993; Izumi *et al.*, 1995), response to growth factors (Feldman *et al.*, 1993; Tajima and Izumi, 1996), and production of growth factors (Sorrell *et al.*, 2004; Mine *et al.*, 2008).

The potential relevance of reticular and papillary fibroblasts with respect to skin aging was recently explored by Mine *et al.* (2008). Two of the most notable markers of aged skin are a decrease of the dermal volume (atrophy) and the loss of rete ridges, the wave-like appearance of the dermal–epidermal junction (Gilchrest and Krutmann, 2006; Makrantonaki and Zouboulis, 2007). It was hypothesized that one of the causes of this aging phenotype is the loss of the papillary dermal compartment. More specifically, it was speculated that papillary fibroblasts (or the papillary phenotype) are lost and replaced by reticular fibroblasts. Since reticular fibroblasts are different from their papillary counterparts, this could cause changes in the skin microenvironment that contribute to aging. Potentially affected attributes include skin elasticity, matrix production and degradation, epidermal interaction, and basement membrane homeostasis. To study these processes in more detail, and to identify the specific role of the fibroblast subpopulations, distinct molecular markers are needed.

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Abbreviations: CCRL1, C–C chemokine receptor type 11; MGP, matrix Gla protein; NTN1, netrin-1; PDPN, podoplanin; TGF-ß1, transforming growth factor-ß1; TGM2, transglutaminase 2; α-SMA, α-smooth muscle actin

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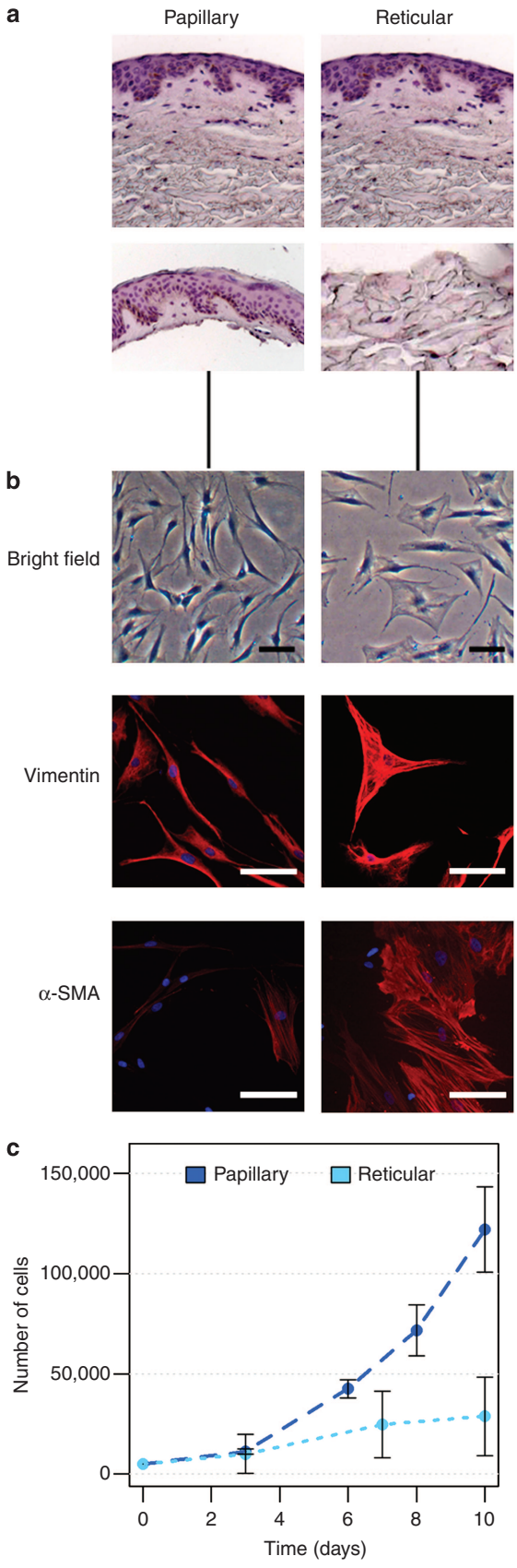

The aim of this study was to identify differentially expressed genes in both subpopulations, and subsequently validate these potential markers at the protein level.

RESULTS

Distinct morphology of papillary and reticular fibroblasts

Cultured reticular and papillary fibroblasts revealed morphological characteristics as described in the literature (e.g. Mine *et al.*, 2008; Sorrell and Caplan, 2009). Papillary fibroblasts exhibit a spindle-shaped morphology, whereas reticular fibroblasts are characterized by a more flattened appearance and have a higher percentage of cells with expression of the myofibroblast marker α-smooth muscle actin (α-SMA). Representative pictures are shown in Figure 1b. In addition, papillary fibroblasts showed increased proliferative capacity (Figure 1c).

Differentially expressed genes in reticular and papillary fibroblasts

Gene expression analysis revealed 116 probes differently expressed in reticular and papillary fibroblasts (adjusted *P*-value <0.05). Figure 2 shows a heatmap of the 50 most significant probes. All significant probes are listed in Supplementary Table S1 online.

In reticular fibroblasts, genes belonging to the smooth muscle contraction pathway were particularly overexpressed, in line with the fact that reticular populations contain more α-SMA-positive fibroblasts. GO term analysis of our data showed that reticular fibroblasts contain predominantly genes involved in cytoskeletal organization, cell motility, and neuronal development. Papillary fibroblasts showed a high expression level of genes belonging to the complement activation pathway, indicating an implication in the skin immune response system. This was confirmed by investigating the GO terms, which showed enrichment for immune response, host defense, and complement activation.

Three reference genes were selected by quantitative reverse transcriptase–PCR (qPCR) following GeNorm analysis: SND1, TBP, and EI24. In total, 16 significantly different genes from the gene expression data were chosen to be validated by qPCR. Of these, 13 were also significantly different in the qPCR analysis (Figure 3).

Figure 1. Culture of papillary and reticular fibroblasts. (**a**) Schematic representation of the isolation of reticular and papillary fibroblasts. For papillary fibroblasts, the epidermis and part of the upper dermis are dermatomed (300 μm). Reticular fibroblasts are taken from the deep dermis by dermatoming at 700 μm and using the dermis beneath the dermatomed tissue. (**b**) Monolayer characteristics of both fibroblast populations. Differences were found in cell morphology, as determined by bright-field microscopy and vimentin staining (intermediate filament). Furthermore, reticular fibroblasts have a higher expression of α-smooth muscle actin (α-SMA). Scale bar = 50 μm. (**c**) Growth curve of reticular and papillary fibroblasts. Points show the average number of cells from three different donors. Reticular fibroblasts grow significantly slower than papillary fibroblasts, as determined by a paired *t*-test at day 10 ($P<0.05$, *t*-test, three different donors).

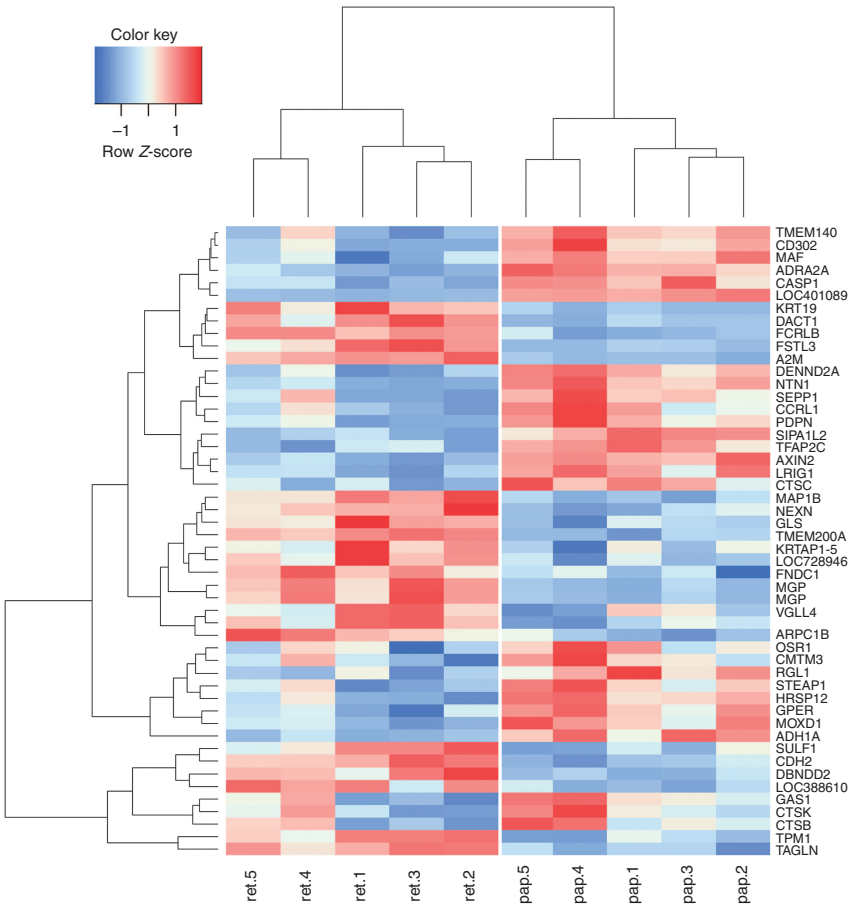

Figure 2. Heatmap of 50 most significant probes. On the *x* axis, the arrays are listed, pap or ret designating the cell type, and the numbers indicate the donor.

Validation of biomarkers on protein level

Of the genes that were confirmed by qPCR, five were selected for further validation at the protein level. These were CCRL1 (C–C chemokine receptor type 11), MGP (matrix Gla protein), NTN1 (netrin-1), PDPN (podoplanin), and TGM2 (transglutaminase 2). The selection was based on a high LogFC and expression at the cell surface (except TGM2, which is expressed intracellularly). Only two of these, TGM2 and PDPN, could be validated by western blot analyses on cell lysates of the same donors used in the array experiment (Figure 4a). The antibodies for the other targets did not work with this approach. Furthermore, CCRL1 did not show any difference in expression (data not shown). Next, we wanted to confirm the expression of TGM2 and PDPN in monolayer cultures of reticular and papillary fibroblasts. PDPN showed strong expression in papillary fibroblasts and weak staining in reticular fibroblasts, whereas TGM2 was expressed in most, but not all, reticular fibroblasts and only occasionally in papillary fibroblasts (Figure 4b). For the other targets, we tried immunohistochemical analyses on *in vivo* paraffin sections of female donors. As expected from our gene expression data, MGP was strongly expressed and abundantly present in the reticular dermal matrix (Figure 5). The MGP-negative band underneath the epidermis decreased in size in aged donors, suggesting a loss of papillary dermis during aging. Although

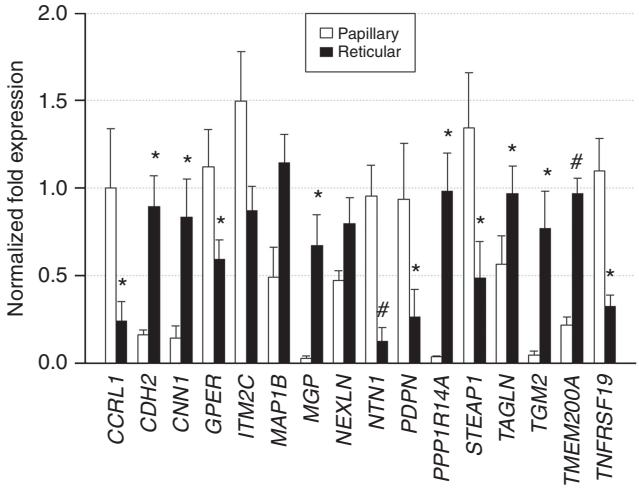

Figure 3. Quantitative PCR validation. Analyses showing that 13 out of 16 target genes were also significantly expressed by PCR. The tested genes are shown on the *x* axis and the normalized fold expression on the *y* axis. The normalized fold expression is based on the reference genes *SND1*, *TBP*, and *EI24*. Error bars show SEM. $^{*}P<0.05$, $^{\#}P<0.01$ (paired *t*-test).

the antibodies tested for NTN1 and PDPN showed background staining in the epidermis, both proteins were highly expressed in singular cells in the upper dermis (Figure 6).

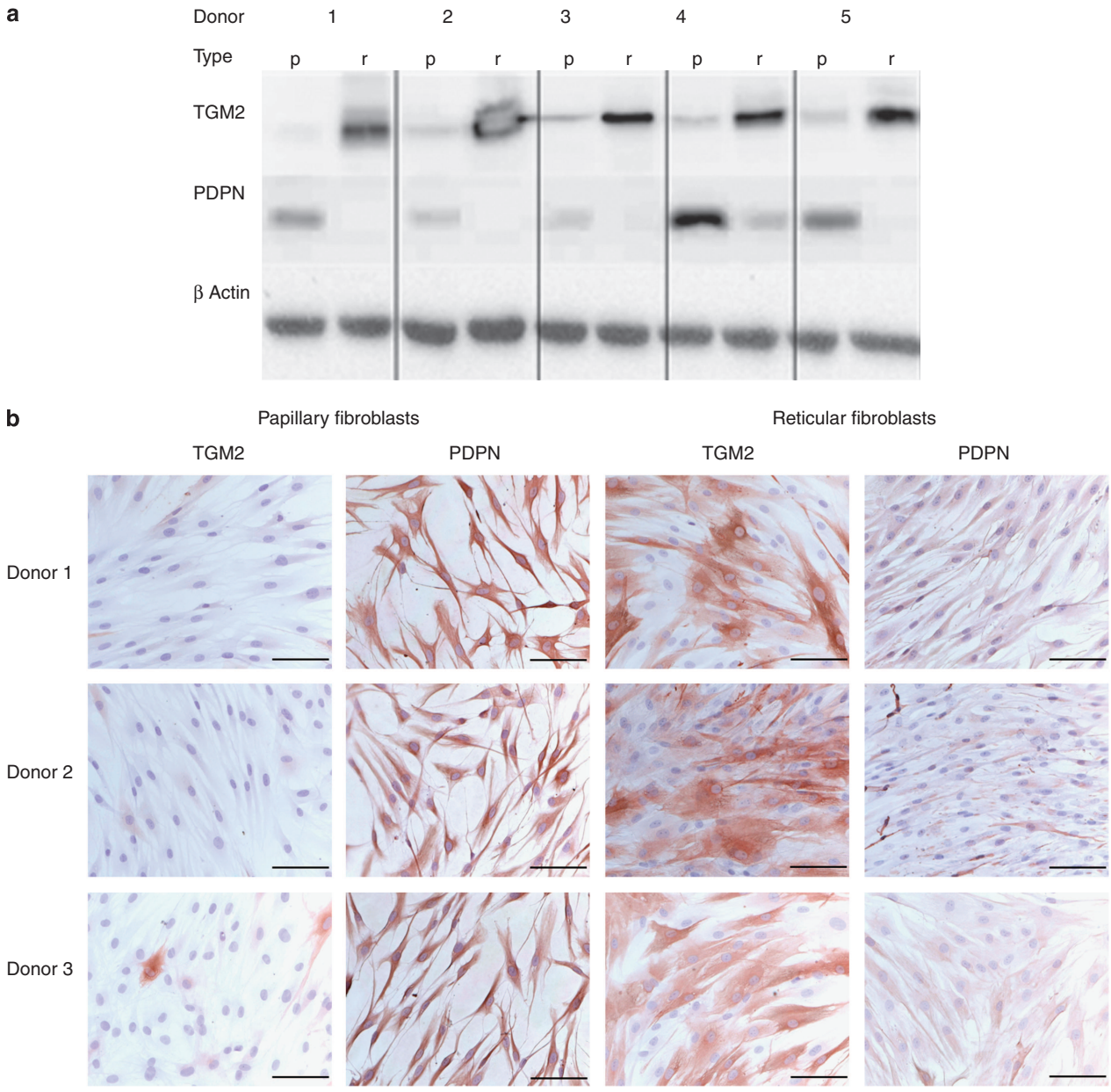

Figure 4. *In vitro* validation of markers on protein level. (**a**) Western blot analyses showing that transglutaminase 2 (TGM2) and podoplanin (PDPN) are also differently expressed between reticular and papillary fibroblasts. Donor designates the donor number and the type of fibroblast subpopulation (p = papillary; r = reticular). (**b**) Immunohistochemical analyses on monolayer cultures of papillary and reticular fibroblasts isolated from three different donors. TGM2 was strongly expressed in reticular fibroblasts and PDPN was more expressed in papillary fibroblasts. Scale bar = 50 μm.

However, there were also cells in the deeper dermis that were positive and negative cells in the upper dermis (Figure 6b). As such, they are not very suitable to differentiate between the papillary and reticular dermis, as there is no clear boundary between positively stained fibroblasts and negatively stained fibroblasts. The target CCRL1 could not be validated on protein level in fibroblasts cultures and *in vivo* biopsies, while TGM2 showed a highly variable expression in *in vivo* biopsies.

DISCUSSION

Two antibodies that selectively detect the papillary dermis have been characterized before (Sorrell *et al.*, 1999, 2003). However, no specific biomarkers for either the papillary or reticular fibroblasts themselves have been described. By using microarray analysis, we have found 116 genes that are differentially expressed in reticular and papillary fibroblasts. Two genes (*PDPN* and *TGM2*) were validated at the protein level in monolayer fibroblast cultures. The most promising marker for reticular fibroblasts was MGP, almost exclusively expressed in the reticular dermis *in vivo*. Even though PDPN and NTN1 showed generally a higher expression in *in vivo* papillary fibroblasts, their use as *in vivo* markers seemed limited because of the highly variable staining.

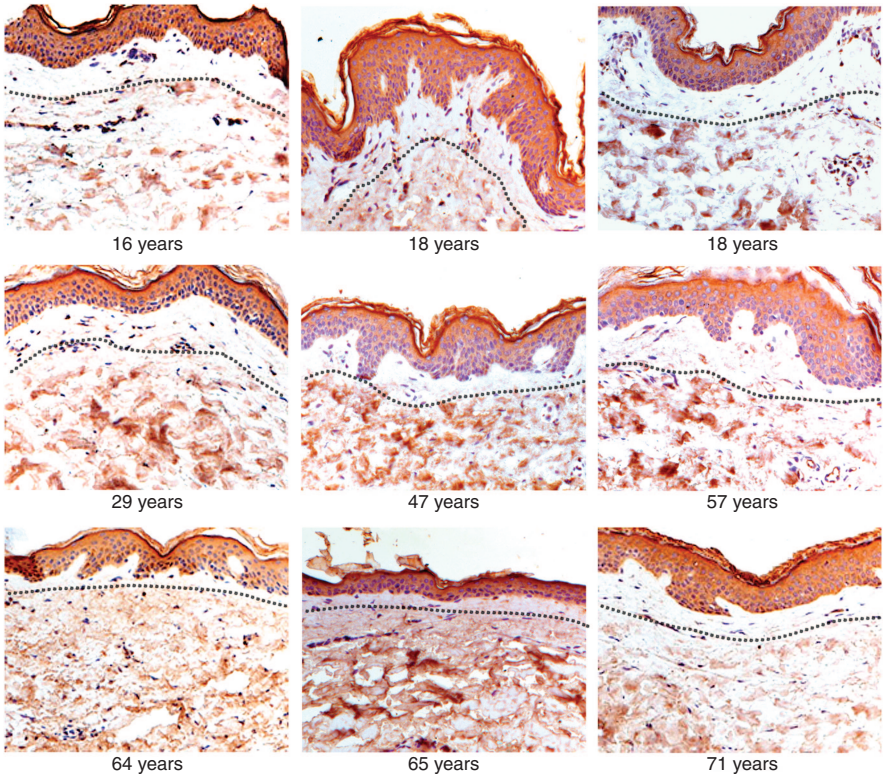

Figure 5. Immunohistochemical analyses of the reticular marker matrix Gla protein (MGP) on *in vivo* sections from mammary and abdominal skin of nine donors. Although the antibody against MGP shows some background staining in the epidermis, a high expression in the reticular dermis and weak or no expression in the papillary dermal layer could be observed. This indicates that this marker can clearly separate between both dermal layers. Scale bar = 50 μm.

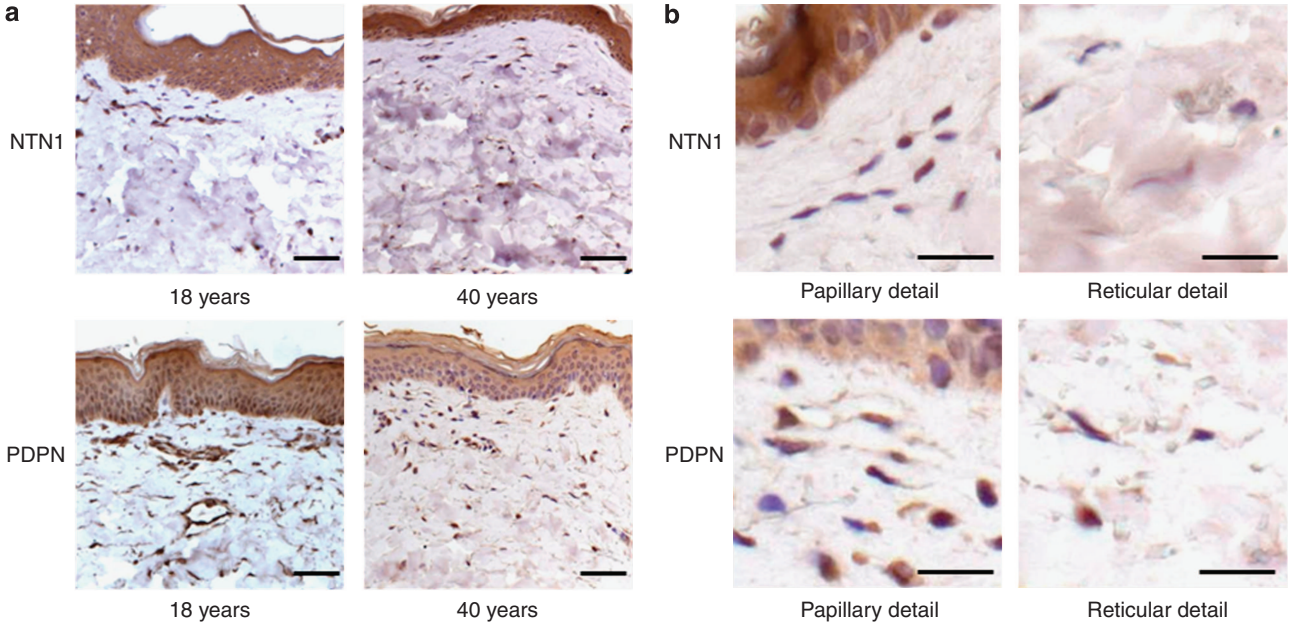

Figure 6. Protein expression of papillary markers netrin-1 (NTN1) and podoplanin (PDPN) *in vivo*. (**a**) Immunohistochemical analyses of the NTN1 and PDPN in *in vivo* sections of two donors (mammary skin). (**b**) High magnification pictures of the papillary and reticular dermis. Although both antibodies show staining in the epidermis, both proteins were found mainly expressed in the fibroblasts underneath the epidermis. However, some fibroblasts stained positive for these antibodies in the reticular dermis, while some fibroblasts stained negative for these antibodies in the papillary dermis. Scale bar = (**a**) 50 μm and (**b**) 25 μm.

One of our objectives was the identification of markers that could be used to separate the two fibroblast populations. Most of the markers we found were not exclusively expressed in one of the populations, neither *in vivo* nor in cultures. This begs the question whether the two populations are homogenous. Earlier findings showed that within the fibroblast populations there is heterogeneity (Sorrell *et al.*, 2007). Even though the markers we identified are useful to determine the phenotype of a fibroblast population, they are less specific if only a few cells are available.

The exact role of the genes found differently expressed between reticular and papillary fibroblasts remains unknown. Some genes could be grouped based on their similar functions, but no individual signaling pathways were found that were clearly distinct. This could be caused by the relative small sample size and the low number of significant genes to work with in the functional analysis. However, based on the GO terms, it appears that reticular fibroblasts show increased expression of genes involved in cell motility and contraction. In our experience, reticular fibroblasts show more contraction in human skin equivalents than papillary fibroblasts. Several validated, reticular markers are associated with myofibroblasts: calponin 1, PPP1R14A, and transglutaminase 2 (Tomasek *et al.*, 2006; Huang *et al.*, 2009; Kulkarni *et al.*, 2011). In addition, TGM2 is linked to transforming growth factor-ß1 (TGF-ß1) activation (Cao *et al.*, 2012). In turn, TGF-ß1 is associated with matrix production and myofibroblast differentiation. This is similar to two properties of the reticular population: high matrix density and an increased number of α-SMA-positive cells. Accordingly, TGF-ß1 may be an important factor involved in the differentiation between papillary and reticular fibroblasts.

The increase of α-SMA-positive fibroblasts in the reticular population could be explained in several, non-exclusive ways. First, reticular fibroblasts contain more α-SMA-positive cells to begin with. It has been shown that deep dermal fibroblasts contain more α-SMA (myofibroblasts) and have increased TGF-ß1 production (Wang *et al.*, 2008). Second, the reticular fibroblasts represent a more differentiated state than papillary fibroblasts and consequently express more fibroblast differentiation markers, such as α-SMA (Bayreuther *et al.*, 1988; Izumi *et al.*, 1995; Mine *et al.*, 2008). And finally, it is possible that reticular fibroblasts have a greater propensity to differentiate into α-SMA-positive cells *in vitro*. This, in turn, could be caused by greater TGF-ß1 production and greater responsiveness to TGF-ß1 of reticular fibroblasts.

MGP was the only gene that showed a strong differential expression *in vivo*. Its function in the skin is unknown, although it is believed to play a role in (inhibition of) calcification of matrix molecules (Davies *et al.*, 2006; Gheduzzi *et al.*, 2007). Interestingly, MGP shows intense staining in the terminally differentiated layers of the epidermis (Figure 5). It is possible that MGP is involved in the density of the tissue (Cancela *et al.*, 1997); MGP expression is increased in dense tissues. This corresponds to the fact that the reticular dermis is denser than the papillary dermis. In addition, it can be an explanation for the lack of differential protein levels of MGP between monolayer cultures of papillary and reticular cultures, because these experiments were not performed on high-density cultures.

The papillary markers were enriched for genes involved in the immune response. Since papillary markers are close to the epidermis, and therefore the outside world, it is likely that they play a role in host defense. However, no clear pathway or process in host defense could be singled out among the genes that showed increased expression in papillary fibroblasts.

Countless questions remain regarding the role of these fibroblast populations in skin physiology, especially their role in relation to skin aging. One of the most prominent markers of skin aging is the loss of rete ridges and the flattening dermis. Mine *et al.* (2008) showed that fibroblasts in the papillary dermis are more affected by aging and hypothesized that the papillary dermis and fibroblasts disappear during aging. This leads to a (relative) increase in the reticular volume and could explain, based on the *in vitro* differences between reticular and papillary fibroblasts, certain aspects of skin aging. One of the markers we have found, MGP, confirms the increase of papillary atrophy with age and a reduced cell density. These findings were most striking in donors of post-menopausal age.

An interesting question is what causes the loss of the papillary dermis in aged skin. UV seems an obvious candidate, but the phenotype is also found in unexposed skin. Dermabrasion of (usually photo-) aged skin rejuvenates it, including a restoration of the papillary dermis (Freedman *et al.*, 2001). This suggests that aged skin is still capable of generating a papillary dermis, but that this is somehow suppressed. Stimulating the skin to (re)generate the papillary dermis looks like a promising way to combat skin aging.

In conclusion, we have identified several markers to distinguish fibroblasts from the papillary and reticular dermis. They can be used *in vitro* on both RNA and protein level, and *ex vivo* for immunohistochemical analysis, in particular MGP. The identified markers may prove useful in further functional studies of the populations and their respective role in skin aging.

MATERIALS AND METHODS

Isolation and cell culture

Five female, Caucasian donors aged 39–49 years were used for the isolation of the fibroblasts. Of all donors, both reticular and papillary were isolated. Consequently, all analyses were performed on a pairwise basis. Isolation was performed as described in the literature (Sorrell *et al.*, 2004; Mine *et al.*, 2008). In short, skin obtained from plastic surgery (mamma reduction or abdominal correction) was cleaned thoroughly and dermatomed at two different depths. First, a 300 μm piece was taken, containing the epidermis and papillary dermis. For the reticular dermis, the skin was dermatomed at 700 μm, and the upper part was discarded. The remaining (deep) dermis was used for fibroblast isolation. A schematic overview of this procedure is given in Figure 1a. Fibroblasts were isolated by treatment with collagenase (Invitrogen, Breda, The Netherlands)/dispase (Roche Diagnostics, Almere, The Netherlands) (3:1) for 2 hours at 37 °C. The cells were subsequently filtered with a 70 μm cell strainer and cultured in DMEM medium (Invitrogen) containing 5% fetal calf serum (HyClone/Greiner, Nürtingen, Germany) and 1% penicillin–streptomycin (Invitrogen). They were kept at 37 °C at 5% CO_2. Fibroblasts used for experiments were in passages 4–6.

Patient consent was not required, because the use of surplus material obtained in accordance with the Dutch Law on Medical Treatment Agreement does not require patient consent.

Growth curve

For the growth curve experiment, 5,000 fibroblasts papillary or reticular fibroblasts of three different donors were seeded into 6-well plates. Cells were counted with a Bürker-counting chamber after 3, 6, 7, and 10 days.

RNA and protein isolation

RNA and proteins were isolated from monolayer fibroblast cultures with the RNEasy kit (Qiagen) and Mammalian Protein Extraction Reagent (M-PER; Thermo Scientific, Etten-Leur, The Netherlands), respectively, according to the manufacturer's instructions. All following experiments were performed with RNA and proteins from a single isolation.

Microarrays

Gene expression analysis was performed by ServiceXS (Leiden, The Netherlands). The platform was Illumina HumanHT-12 Expression BeadChip. Data were generated with the Beadstudio software of Illumina and analysis was performed in R (2.10.0). For the analysis, the data were imported and normalized with the *lumi* package (Robust Spline Normalization) (Du *et al.*, 2008) and followed by the expression analysis with the *limma* package (Smyth, 2004). Probes that showed no expression in all of the arrays (detection *P*-value >0.05) were not included in the analysis. For multiple testing correction, the false discovery rate method was used (Benjamini and Hochberg, 1995).

Pathway analysis

Pathway and GO term enrichment analysis was performed with the DAVID tool (Dennis Jr *et al.*, 2003). Two lists were uploaded: one with genes that were upregulated in reticular fibroblasts (adj. *P*-value <0.1 and LogFC >0.7) and one with genes upregulated in papillary fibroblasts (adj. *P*-value <0.1 and LogFC <-0.7). Both lists had approximately 80 genes.

QRT-PCR

cDNA was generated from 1 μg RNA using the iScript cDNA synthesis kit (BioRad, Veenendaal, The Netherlands) according to manufacturer's instructions. PCR reactions were based on the SYBR Green method (BioRad). The PCRs were run on the MyIQ system (BioRad). The PCR cycles were: 3.5 minutes at 95 °C to activate the polymerase, 35 cycles of 20 seconds at 95 °C and 40 seconds at 60 °C, followed by the generation of a melt curve. Primers were checked before on a dilution series of normal fibroblast cDNA. Reference genes were analyzed with the GeNorm method (Vandesompele *et al.*, 2002). Expression analysis was performed with the BioRad software (iQ5) and was based on the delta delta Ct method with the reference genes that were most stably expressed in the GeNorm analysis. The primers are listed in Supplementary Table S2 online.

Western blot

In all, 7 μg of each protein sample was added to the loading buffer, heated to 90 °C for 5 minutes, and loaded onto a 10% SDS-PAGE gel. Proteins were blotted on a polyvinylidene difluoride membrane (Thermo Scientific). Blocking was performed with 5% Protifar Plus (Nutricia, Zoetermeer, The Netherlands) in phosphate-buffered saline-T (0.1% Tween). Primary antibodies were incubated overnight at 4 °C. Thereafter, membranes were incubated with the appropriate secondary antibodies, either stabilized horseradish peroxidase–conjugated anti-mouse or anti-rabbit (Thermo Scientific; dilution 1:1,500). For detection of the bands, the Supersignal West Femto ECL system (Thermo Scientific/Pierce) was applied to the membrane. Bands were visualized using the G-box technology and software.

Immunohistochemical analyses

For immunohistochemical analyses on monolayer cell cultures, fibroblasts were grown on glass slides until nearly confluent, washed in phosphate-buffered saline, and fixed with 4% formaldehyde. Staining was visualized by a secondary antibody with a fluorescent dye (Cy3). 4′,6-Diamidino-2-phenylindole was used as counterstain. Immunohistochemical analysis was performed on paraffin-embedded *in vivo* skin sections. Slides (5 μm thick) were cut, deparaffinized, rehydrated, and washed with phosphate-buffered saline. Heat-mediated antigen retrieval at pH 6 was performed, followed by a block of endogenous peroxidase and a block step using phosphate-buffered saline/1% bovine serum albumin/2% normal human serum. Primary antibodies were incubated overnight at 4 °C. Staining was visualized using BrightVision+ poly-horseradish peroxidase (Immunologic, Duiven, The Netherlands) according to the manufacturer's instructions, and 3,3′ diaminobenzidine as a chromogen. Counterstaining was performed with hematoxylin.

Antibodies

The antibodies used in this study were: α-SMA (1A4; Sigma, Zijndrecht, The Netherlands) 1:800, CCRL1 (ab74806; Abcam, Cambridge, UK) 1:1,000, MGP (A-11; Santa Cruz) 1:100, NTN-1 (H-104; Santa Cruz, Santa Cruz, CA) 1:75, PDPN (18H5; Abcam) 1:1,000 (WB) and 1:100 (IHC), TGM2 (CUB7402; Abcam) 1:1,000 (WB) and 1:100 (IHC), and vimentin (V9; AbD Serotec, Düsseldorf, Germany) 1:50.

CONFLICT OF INTEREST

GS and CM are employees of CHANEL Parfum Beauté. AEG declares the receipt of a grant from CHANEL. The other authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Azzarone B, Macieira-Coelho A (1982) Heterogeneity of the kinetics of proliferation within human skin fibroblastic cell populations. *J Cell Sci* 57:177–87

Bayreuther K, Rodemann HP, Hommel R *et al.* (1988) Human skin fibroblasts *in vitro* differentiate along a terminal cell lineage. *Proc Natl Acad Sci USA* 85:5112–6

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate—a practical and powerful approach to multiple testing. *J R Statist Soc Ser B* 57:289–300

Cancela ML, Hu B, Price PA (1997) Effect of cell density and growth factors on matrix GLA protein expression by normal rat kidney cells. *J Cell Physiol* 171:125–34

Cao L, Shao M, Schilder J *et al.* (2012) Tissue transglutaminase links TGF-beta, epithelial to mesenchymal transition and a stem cell phenotype in ovarian cancer. *Oncogene* 31:2521–34

Chang HY, Chi JT, Dudoit S *et al.* (2002) Diversity, topographic differentiation, and positional memory in human fibroblasts. *Proc Natl Acad Sci USA* 99:12877–82

Davies CA, Jeziorska M, Freemont AJ *et al.* (2006) Expression of osteonectin and matrix Gla protein in scleroderma patients with and without calcinosis. *Rheumatology (Oxford)* 45:1349–55

Dennis G Jr, Sherman BT, Hosack DA *et al.* (2003) DAVID: database for annotation, visualization, and integrated discovery. *Genome Biol* 4:3

Du P, Kibbe WA, Lin SM (2008) Lumi: a pipeline for processing illumina microarray. *Bioinformatics* 24:1547–8

Feldman SR, Trojanowska M, Smith EA *et al.* (1993) Differential responses of human papillary and reticular fibroblasts to growth factors. *Am J Med Sci* 305:203–7

Freedman BM, Rueda-Pedraza E, Waddell SP (2001) The epidermal and dermal changes associated with microdermabrasion. *Dermatol Surg* 27:1031–3

Gheduzzi D, Boraldi F, Annovi G *et al.* (2007) Matrix Gla protein is involved in elastic fiber calcification in the dermis of pseudoxanthoma elasticum patients. *Lab Invest* 87:998–1008

Gilchrest BA, Krutmann J (2006) *Skin aging*. Berlin, Heidelberg: Springer, 198pp

Harper RA, Grove G (1979) Human skin fibroblasts derived from papillary and reticular dermis: differences in growth potential *in vitro*. *Science* 204:526–7

Huang L, Haylor JL, Hau Z *et al.* (2009) Transglutaminase inhibition ameliorates experimental diabetic nephropathy. *Kidney Int* 76:383–94

Izumi T, Tajima S, Nishikawa T (1995) Differential expression of alpha 1 and alpha 2 chains of type VI collagen in the upper, middle, and lower dermal fibroblasts *in vitro*. *J Biochem* 117:1004–7

Kalluri R, Zeisberg M (2006) Fibroblasts in cancer. *Nat Rev Cancer* 6:392–401

Kulkarni AA, Thatcher TH, Olsen KC *et al.* (2011) PPAR-gamma ligands repress TGFbeta-induced myofibroblast differentiation by targeting the PI3K/Akt pathway: implications for therapy of fibrosis. *PLoS One* 6:e15909

Maier AB, Westendorp RG (2009) Relation between replicative senescence of human fibroblasts and life history characteristics. *Ageing Res Rev* 8:237–43

Makrantonaki E, Zouboulis CC (2007) Molecular mechanisms of skin aging: state of the art. *Ann N Y Acad Sci* 1119:40–50

Mine S, Fortunel NO, Pageon H *et al.* (2008) Aging alters functionally human dermal papillary fibroblasts but not reticular fibroblasts: a new view of skin morphogenesis and aging. *PLoS One* 3:e4066

Rinn JL, Bondre C, Gladstone HB *et al.* (2006) Anatomic demarcation by positional variation in fibroblast gene expression programs. *PLoS Genet* 2:e119

Rinn JL, Wang JK, Liu H *et al.* (2008) A systems biology approach to anatomic diversity of skin. *J Invest Dermatol* 128:776–82

Ross MH, Pawlina W (2011) *Histology: a text and atlas*. Lippincott Williams & Wilkins, 928pp

Schonherr E, Beavan LA, Hausser H *et al.* (1993) Differences in decorin expression by papillary and reticular fibroblasts *in vivo* and *in vitro*. *Biochem J* 290(Part 3):893–9

Smyth GK (2004) Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 3; Article3

Sorrell JM, Baber MA, Brinon L *et al.* (2003) Production of a monoclonal antibody, DF-5, that identifies cells at the epithelial–mesenchymal interface in normal human skin. APN/CD13 is an epithelial–mesenchymal marker in skin. *Exp Dermatol* 12:315–23

Sorrell JM, Baber MA, Caplan AI (2004) Site-matched papillary and reticular human dermal fibroblasts differ in their release of specific growth factors/cytokines and in their interaction with keratinocytes. *J Cell Physiol* 200:134–45

Sorrell JM, Baber MA, Caplan AI (2007) Clonal characterization of fibroblasts in the superficial layer of the adult human dermis. *Cell Tissue Res* 327:499–510

Sorrell JM, Caplan AI (2004) Fibroblast heterogeneity: more than skin deep. *J Cell Sci* 117:667–75

Sorrell JM, Caplan AI (2009) Fibroblasts—a diverse population at the center of it all. *Int Rev Cell Mol Biol* 276:161–214

Sorrell JM, Carrino DA, Baber MA *et al.* (1999) A monoclonal antibody which recognizes a glycosaminoglycan epitope in both dermatan sulfate and chondroitin sulfate proteoglycans of human skin. *Histochem J* 31:549–58

Tajima S, Izumi T (1996) Differential *in vitro* responses of elastin expression to basic fibroblast growth factor and transforming growth factor beta 1 in upper, middle and lower dermal fibroblasts. *Arch Dermatol Res* 288:753–6

Tajima S, Pinnell SR (1981) Collagen synthesis by human skin fibroblasts in culture: studies of fibroblasts explanted from papillary and reticular dermis. *J Invest Dermatol* 77:410–2

Tomasek JJ, Vaughan MB, Kropp BP *et al.* (2006) Contraction of myofibroblasts in granulation tissue is dependent on Rho/Rho kinase/myosin light chain phosphatase activity. *Wound Repair Regen* 14:313–20

Vandesompele J, De PK, Pattyn F *et al.* (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3:RESEARCH0034-11

Wang J, Dodd C, Shankowsky HA *et al.* (2008) Deep dermal fibroblasts contribute to hypertrophic scarring. *Lab Invest* 88:1278–90