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# Culture Medium and Cell Density Impact Gene Expression in Normal Skin and Abnormal Scar-Derived Fibroblasts

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Fibroblasts, the main cell type of the dermis, are responsible for production and remodeling of extracellular matrix during wound healing. Disruption of either production or degradation of extracellular matrix can lead to abnormal scarring, resulting in hypertrophic scar or keloid scar. Aberrations in proliferation and gene expression have been observed in fibroblasts isolated from abnormal scars, but differences observed may be related to biologic responses to growth conditions and media formulations. This study examined gene expression in primary human fibroblasts from normal skin or abnormal scar in two culture media formulations and three relative cell densities. In general, higher expression of collagen type 1 *alpha-1* (*COL1A1*) and *alpha-2* (*COL1A2*) and matrix metalloproteinase 3 (*MMP3*) and lower levels of *MMP1* were observed in all cell strains cultured in standard medium containing 10% fetal bovine serum compared with cells cultured in medium optimized for proliferation. Normal and scar-derived fibroblasts exhibited differences in gene expression in specific response to media formulations and cell density. *COL1A1* and *COL1A2* were increased, and *MMP1* and *MMP3* were decreased, in keloid cells compared with normal fibroblasts under most conditions analyzed. However, expression of *plasminogen activator inhibitor 1* in keloid fibroblasts, which was significantly different than in normal fibroblasts, was either increased or decreased in response to the medium formulation and relative cell density. A related gene, *plasminogen activator inhibitor 2*, was shown for the first time to be significantly increased in keloid fibroblasts compared with normal fibroblasts, in both media formulations and at all three cell densities. The results emphasize the critical role of culture conditions in interpretation of cell behavior and expression data and for comparison of cells representing normal and fibrotic phenotypes. (J Burn Care Res 2011;32:498–508)

Fibroblasts are the main cell type of the dermis and are responsible for production and remodeling of extracellular matrix (ECM) in intact skin and during wound healing. Disruption of the balance between synthesis and degradation of ECM during the remodeling phase of wound healing can lead to abnormal

scarring, resulting in hypertrophic scar (HTS) or keloid scar. Hypertrophic and keloid scarring are both debilitating and disfiguring fibroproliferative disorders that can occur after burns and other skin injuries and share some common features. These include, but are not limited to, excessive collagen deposition caused by unknown mechanisms; significantly impaired function because of itching, pain, and decreased range of motion<sup>1</sup>; profoundly impacted psychosocial well-being; and impaired overall quality of life for affected patients.<sup>1–4</sup> HTSs are confined to the original wound area, grow intensely for a finite period, and then often regress.<sup>3–5</sup> Keloids are distinguished from HTS in that they extend beyond the margin of the original wound, tend to proliferate indefinitely, and often recur after excision.<sup>5–8</sup> Importantly, there are no real cures for either type of scar, despite the availability

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of multiple treatment alternatives.<sup>5,6,9</sup> The fact that there are no universally accepted treatments for either keloids or HTS indicates that the key mechanisms involved in abnormal scarring remain to be elucidated.

Fibroblasts in intact keloid scars were reported to have a higher proliferative activity compared with cells in HTS or normal skin,<sup>10</sup> and cultured keloid fibroblasts were found to have a greater proliferative response to in vitro wounding compared with fibroblasts from normal skin,<sup>11</sup> suggesting that increased proliferation may contribute to dysregulated growth in abnormal scars. Gene expression studies have described differential expression of specific genes involved in regulation of ECM in HTS and keloids compared with normal skin, and aspects of these expression profiles are maintained in cultured fibroblasts isolated from scar tissue.<sup>12-16</sup> Abnormal collagen accumulation is a feature of both HTS and keloids, and in particular, type I collagen expression is elevated in fibroblasts of HTS and keloids. This has been attributed to increased transcription of the genes encoding the alpha-1 (*COL1A1*) and alpha-2 (*COL1A2*) chains of type I collagen, although variable levels of elevated expression have been reported.<sup>17-21</sup> Increased ECM deposition in HTS and keloids also results from decreased degradation of collagen and other matrix components because of decreased protease expression. For example, expression of matrix metalloproteinase I (*MMPI*), also known as interstitial collagenase, is decreased in fibroblasts from HTS.<sup>22-24</sup> *MMPI* is involved in degradation of mainly types I, II, and III collagen. *MMP3*, or stromelysin, degrades fibronectin, laminin, and collagens III, IV, IX, and X. Fibronectin expression is increased in fibroblasts from keloid scars,<sup>25,26</sup> and expression of *MMP3* is decreased,<sup>15,27</sup> leading to overproduction of fibronectin. Reduced breakdown of the provisional fibrin matrix also characterizes many fibrotic skin diseases. The serine protease inhibitor (serpin) *plasminogen activator inhibitor 1* (*PAI-1*), which is highly expressed in keloid fibroblasts,<sup>28,29</sup> is the major inhibitor of urokinase-type plasminogen activator (uPA); uPA converts plasminogen into plasmin, which in turn breaks down fibrin.<sup>30</sup> *PAI-1* is a downstream target of transforming growth factor beta 1 (*TGF-β1*), a profibrotic growth factor that is increased in abnormal scars and regulates collagen levels in keloid fibroblasts.<sup>28,29,31-33</sup> *TGF-β1* has been implicated in other fibrotic diseases, such as systemic sclerosis,<sup>34</sup> and *PAI-1* is also increased in scleroderma fibroblasts.<sup>35</sup> Interestingly, the serpin *PAI-2* is also highly increased in scleroderma fibroblasts, where it is induced by factors present in serum but not *TGF-β1*.<sup>36</sup> *PAI-2*

expression in HTS or keloid fibroblasts has not been previously described.

In many previous gene expression studies, fibroblasts from normal skin or scar tissue were cultured in media containing high levels of fetal bovine serum (FBS) and/or were cultured to confluence, factors that can influence gene expression and proliferation. Differences in culture conditions may explain some discrepancies in expression data reported in studies of abnormal scar-derived cells. For example, Ala-Kokko et al<sup>37</sup> found no significant increase in collagen synthesis between normal and keloid fibroblasts, whereas Uitto et al<sup>17</sup> demonstrated a 2-fold increase in type I procollagen production and a mean 1.7-fold increase in *COL1A2* mRNA. In the latter study, the fibroblasts were cultured in medium containing 10% FBS; the former study used medium supplemented with 10% FBS + ascorbic acid. Interestingly, ascorbic acid is known to increase collagen production,<sup>38</sup> suggesting other aspects of the culture environment, such as cell density or passage number, might contribute to the different results obtained in those studies. Expression of genes regulating ECM deposition can vary with time in culture, as was observed for *MMPI* in normal and HTS fibroblasts after multiple serial subcultures.<sup>23</sup>

Our laboratories have been involved in primary culture of human skin cells for development of engineered skin substitutes for grafting in patients with large skin injuries,<sup>39</sup> which requires rapid expansion of large populations of cells from relatively small skin biopsies. For preparation of skin substitutes, media formulations for primary culture of both keratinocytes and fibroblasts have been developed and optimized to produce highly proliferative cells that are also capable of subsequent differentiation.<sup>40,41</sup> Gene expression profiling demonstrated that normal dermal fibroblasts cultured under conditions optimized for proliferation and harvested during log-phase growth express multiple families of genes involved in signal transduction, cell communication, cell growth, and metabolism, as well as ECM,<sup>42</sup> consistent with high proliferation rates. We began to evaluate gene expression in fibroblasts derived from HTS and keloid scars and found that some of our results were inconsistent with previously published data. Hypothetically, differences in gene expression between normal and scar-derived fibroblasts may depend on culture conditions, and may vary if cells are grown in different media formulations, or are analyzed at different cell densities. This study examines the differential effects of culture media formulation and cell density on gene expression in fibroblasts cultured from normal skin, HTS, and keloid scar.

## MATERIALS AND METHODS

### Tissue Sources

Tissue samples (Table 1) were obtained with Institutional Review Board approval and in accordance with the Declaration of Helsinki Principles from patients undergoing plastic and reconstructive surgery procedures at the University Hospital at the University of Cincinnati and at Shriners Hospitals for Children–Cincinnati. For scar samples, clinical diagnosis of keloid vs HTS was made before surgery based on physicians' assessments, which included evaluations of patient history and the degree of overgrowth of scar beyond original wound boundary. Diagnosis was confirmed by examinations of histological sections. All skin samples were trimmed to remove subcutaneous tissue before primary cell isolation.

### Cell Culture

Primary cultures of fibroblasts were established as described in detail elsewhere with minor modifications.<sup>4,3</sup> Briefly, full-thickness skin was washed in 5% Dettol (Reckitt Benckiser, Berkshire, UK), rinsed in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered saline, trimmed to 2 × 0.2 cm strips, and incubated overnight at 4°C in Dispase II (Roche Applied Science, Indianapolis, IN). Epidermal and dermal strips were mechanically separated; dermal pieces were finely minced and digested with collagenase (Worthington Biochemical Corp., Lake-

wood, NJ) for 2 hours at 37°C with periodic agitation. Cells were collected by centrifugation, rinsed, and inoculated into tissue culture flasks in human fibroblast proliferation medium (HFPM) which consisted of Dulbecco's modified Eagle's medium (DMEM, low glucose; Invitrogen, Carlsbad, CA) supplemented with 10 ng/mL epidermal growth factor (EGF; PeproTech, Rocky Hill, NJ), 5 μg/mL insulin (PeproTech), 0.5 μg/mL hydrocortisone (Sigma-Aldrich, St. Louis, MO), 0.1 mM ascorbic acid-2-phosphate (a stable derivative of vitamin C; Sigma-Aldrich), 4% FBS (Invitrogen), and 1× Penicillin-Streptomycin-Fungizone (Invitrogen). After 24 hours, approximately half of cells had adhered to flasks. To increase cell recovery, culture media containing partially digested tissue pieces and nonadherent cells were collected and centrifuged, and the collected cells and tissue pieces were inoculated into fresh flasks. Using this approach, cells that were not released from the dermal tissue by collagenase digestion were not discarded but were allowed time to migrate out of the tissue; all cells were combined before subculturing to passage 1. Media in all flasks were refreshed every 48 and 24 hours before harvesting. Cells were subcultured when they reached ~90% confluence; cells were harvested by incubation for 2 minutes at room temperature with 0.025% trypsin (Sigma-Aldrich) in 0.01% ethylenediaminetetraacetic acid (EDTA) and collection by centrifugation, and at passage 1 were cryopreserved in DMEM + 20% FBS + 10% dimethyl sulfoxide.

For analysis of proliferation and gene expression in different media formulations, fibroblasts from samples 1 (normal skin), 5 (HTS), and 6 (keloid) were used (Table 1). Fibroblasts were recovered from cryopreservation and were inoculated into 75 cm<sup>2</sup> flasks at passage 2 containing HFPM or DMEM + 10% FBS (DFBS) at a density of 2 × 10<sup>3</sup> cells/cm<sup>2</sup>. Media were exchanged every 2 days. Cells were harvested by trypsin/EDTA treatment, as described earlier, at three different time points, representing preconfluent (4 days), confluent (5 days), or postconfluent (8 days) cell densities (n = 3 flasks per group per time point). Cell counts for individual flasks were determined using a hemocytometer. Aliquots of cells (3–4 × 10<sup>6</sup> each) were used for RNA isolation (see below).

### Expression Analyses

Cells for RNA isolation were lysed using Qiashredders (Qiagen, Inc., Valencia, CA), and DNA-free RNA was isolated using the RNeasy Mini Kit combined with the RNase-free DNase system (Qiagen, Inc.). First-strand cDNA was synthesized using 2.5 μg of total RNA using the SuperScript VILO Synthesis Kit (Invitrogen), and quantitative real-time polymerase chain reaction (qRT-

**Table 1.** Tissue samples used for fibroblast isolation

Sample	Type of Scar/Skin	Body Location	Patient Age	Gender	Race
1*	Normal skin	Breast	16 yr	F	AA
2	Normal skin	Foreskin	Newborn	M	C
3	Normal skin	Breast	17 yr	F	AA
4	Normal skin	Abdomen	25 yr	M	AA
5*	HTS	Face	15 yr	M	AA
6*	Keloid	Face	10 yr	M	AA
7	Keloid	Ear			
8†	Keloid	Ear	8 yr	M	C
9†	Keloid	Ear	10 yr	M	C
10	Keloid	Chin	11 yr	M	C
11	Keloid	Ear	17 yr	M	C
12	Keloid	Face	10 yr	M	AA
13	Keloid	Abdomen			

\* Cells from samples 1, 5, and 6 were used for experiments involving different cell densities and media formulations (Figures 1–3). Cells from samples 2–4 and 7–13 were used for analysis of *PAI-2* expression.

† Keloid samples 8 and 9 were collected from the same patient with a 2-yr interval.

F, female; M, male; C, Caucasian; AA, African American.

PCR) was performed using gene-specific RT<sup>2</sup> qPCR Primers and RT<sup>2</sup> SYBR Green/Fluorescein PCR Master Mix (Qiagen, Inc.). RT-PCR amplification was performed using the BIO-RAD iCycler iQ system (BioRad, Hercules, CA). The cycle threshold (CT) values were determined using the iCycle iQ system software (BioRad), and the comparative  $\Delta\Delta CT$  method was used to calculate the fold differences between the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene (*GAPDH*) and the genes of interest (*COL1A1*, *COL1A2*, *MMP1*, *MMP3*, *PAI-1/SERPINE1*, and *PAI-2/SERPIN2*).<sup>44</sup> Triplicate flasks of cells were processed individually for RNA isolation, and each RNA sample was analyzed in technical triplicate for each primer set and *GAPDH*. Values for technical triplicates were averaged, and these values were normalized to expression in normal fibroblasts cultured to postconfluence in DFBS. The results are presented as mean  $\pm$  SE.

For validation of *PAI-2* expression, 10 additional cell strains (normal samples 2–4, keloid samples 7–13; Table 1) were analyzed. Fibroblasts were recovered from cryopreservation and were grown to confluence in HFBM; harvesting, RNA isolation, and qRT-PCR analysis were performed as described earlier. Technical triplicates were performed for each cell strain; the resulting values were averaged, referenced to *GAPDH* control reactions, and normalized to the mean expression levels in the three normal skin fibroblast samples.

### Statistical Analyses

Statistical tests were performed using SigmaStat software version 3.10 (Systat Software, Inc., Chicago, IL). To determine the effects of culture media at different time points, cell count data were analyzed using two-way repeated measures analyses of variance, and pairwise multiple comparison procedures used the Student-Newman-Keuls Method. For analysis of

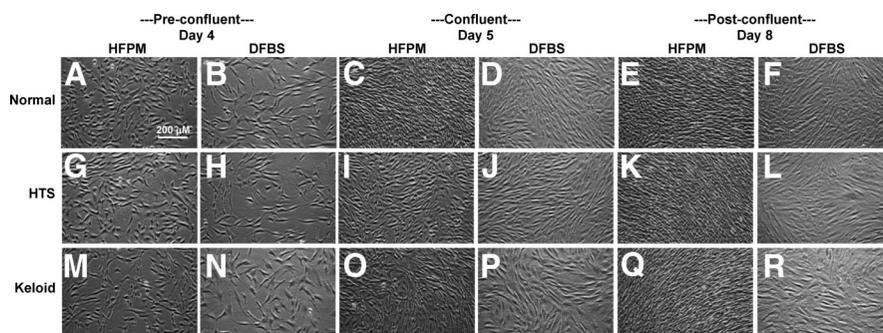
normalized expression levels, pairwise comparisons were performed using *t*-test or Mann-Whitney rank-sum test, as indicated. Significance was established at the 95% confidence level ( $P < .05$ ).

## RESULTS

### Fibroblast Morphology and Proliferation

Primary dermal fibroblasts at passage 2 were cultured in DMEM + 10% FBS (DFBS), which is a standard liquid media formulation for in vitro fibroblast culture, and HFBM, which has been optimized for rapid proliferation of primary fibroblasts. Fibroblasts isolated from normal skin, HTS, and keloid scar were inoculated at equal densities into triplicate flasks that were harvested at three different time points, which approximated subconfluent (50–70%), confluent (90–100%), and postconfluent cell densities based on observation of normal fibroblasts in HFBM (Figure 1).

Fibroblasts cultured in HFBM appeared smaller and displayed a more compact morphology compared with fibroblasts cultured in DFBS (Figure 1). At pre-confluent density (culture day 4), fibroblasts in both media types appeared fusiform or dendritic, but cellular projections appeared longer in DFBS (Figure 1A, B, G, H, M, N). Normal skin fibroblasts reached confluence at culture day 5 in HFBM (Figure 1C), whereas space was still visible in flasks containing HTS and keloid fibroblasts at this time point (Figure 1I, O). Numerous refractive cells were observed at confluence in HFBM. In contrast, fibroblasts in DFBS were larger and flatter (Figure 1C, D, I, J, O, P). At postconfluent densities (culture day 8), fibroblasts in HFBM were densely packed, maintained their relatively small size, and numerous refractive cells were still observed (Figure 1E, K, Q). However, fibroblasts cultured in DFBS formed flattened sheets with few if any refractive cells



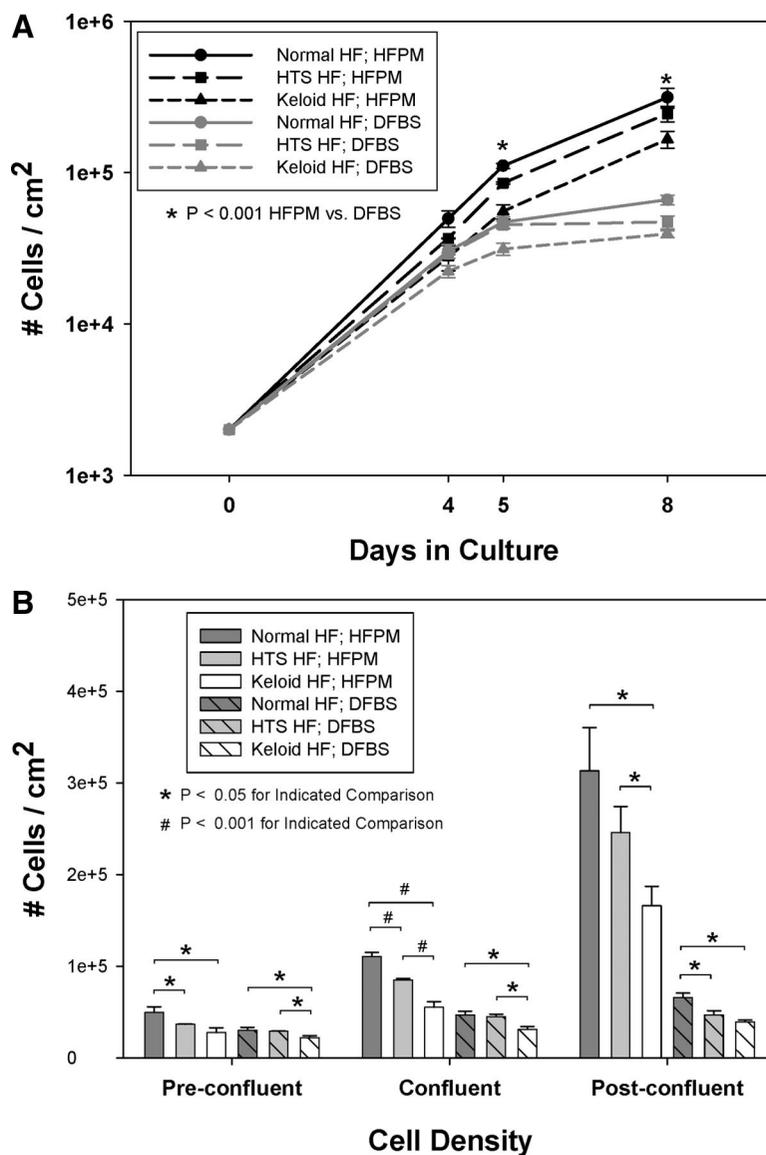
**Figure 1.** Morphology of fibroblasts isolated from normal skin, hypertrophic scar, and keloid scar. Cells were photographed at the same magnification using phase-contrast microscopy. A-F, normal fibroblasts; G-L, HTS fibroblasts; M-R, keloid fibroblasts. Cells were cultured for varying times and using different media formulations, as indicated. Scale bar in (A) is for all panels (200  $\mu$ m).

present (Figure 1F, L, R). Compared with normal or HTS cells, or keloid cells cultured in HFPM, the morphology of keloid-derived fibroblasts in DFBS was heterogeneous, with many cells displaying a flattened appearance at all three cell densities examined (Figure 1M-R).

The trends in cell density that were observed microscopically were confirmed by analysis of cell counts at culture days 4, 5, and 8. For all three fibroblast strains, significantly more cells were obtained at culture days 5 (confluence) and 8 (postconfluence) in HFPM than in DFBS (Figure 2A). In HFPM, continued proliferation was observed postconfluence,

with significantly greater numbers of cells for each fibroblast strain at day 8 compared with day 5 ( $P < .001$ ). In DFBS, cell proliferation postconfluence was observed, but the magnitudes of the changes were lower; significantly more cells were obtained at day 8 than day 5 for normal ( $P = .006$ ) and keloid ( $P = .018$ ) fibroblasts but not HTS fibroblasts ( $P = .555$ ).

Differences in cell counts were observed among cell strains at all three densities examined but were most significant ( $P < .001$ ) in HFPM in confluent fibroblasts. Significantly more normal skin fibroblasts were obtained than keloid fibroblasts at all three cell



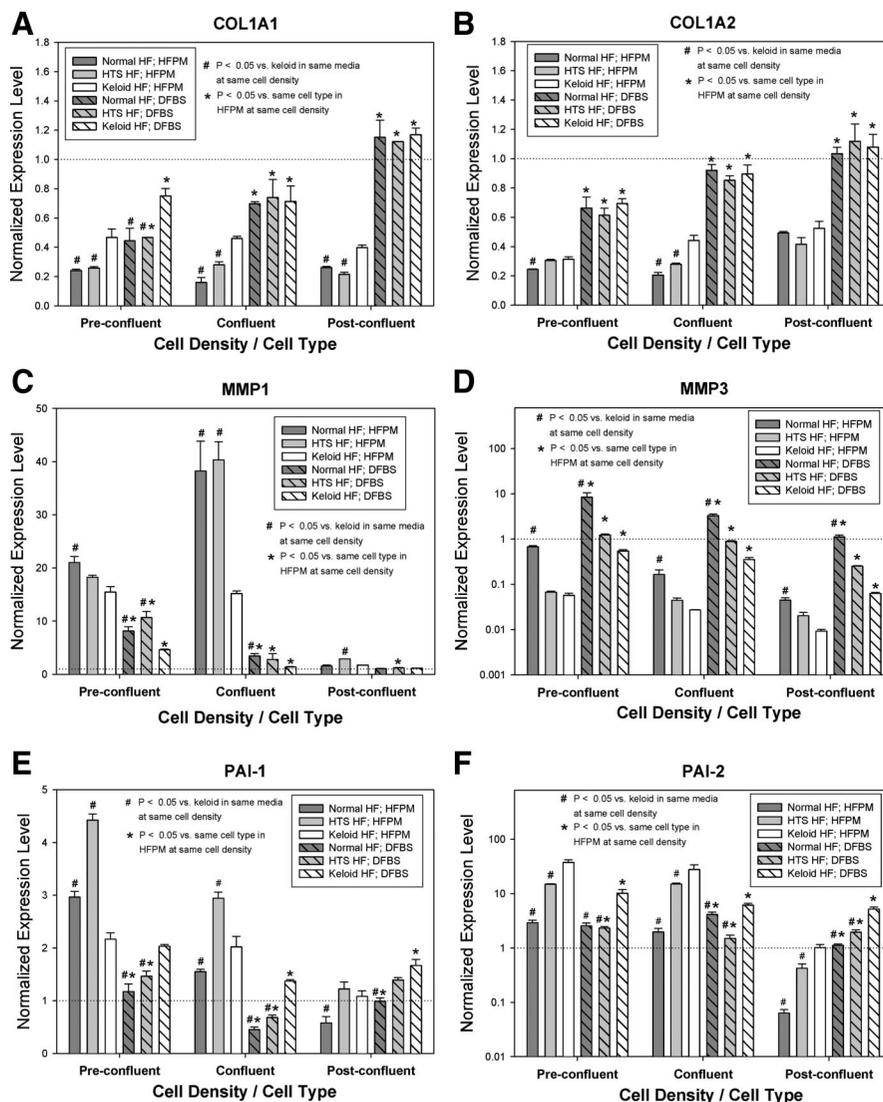
**Figure 2.** Primary human fibroblasts (HF) are more proliferative when cultured in HFPM compared with DFBS. A, Line plots depicting mean cell counts  $\pm$  SEM vs time; significantly greater numbers of human fibroblasts were obtained at days 5 and 8 when cells were cultured in HFPM. B, Bar plots depicting the same data, showing statistically significant differences ( $t$  test) between cell types in the different media conditions and at different relative cell densities, as indicated.

densities and in both types of culture media (Figure 2B). Significant differences between normal and HTS fibroblasts were observed in HFPM media at pre-confluent and confluent densities and in DFBS at post-confluent density. Significant differences in cell count were found between HTS and keloid fibroblasts only in DFBS at preconfluent and confluent densities and in HFPM at postconfluent density (Figure 2B).

### Gene Expression Variations at Different Cell Densities and in Different Media Formulations

Quantitative RT-PCR was used to examine the impact of genotype, culture medium formulation, and

cell density on gene expression in fibroblasts in vitro. Expression levels for *COL1A1* were significantly higher if cells were cultured in DFBS than in HFPM (Figure 3A). Except for *COL1A1* in normal skin fibroblasts at preconfluence, significant differences between media formulations were observed for all three cell strains and cell densities. *COL1A1* expression in fibroblasts cultured in DFBS was significantly impacted by cell density: in normal and HTS fibroblasts, *COL1A1* was significantly higher in confluent cells compared with preconfluent cells ( $P < .05$ ) and was significantly higher for all three cell strains in post-confluent compared with confluent cells ( $P < .001$ ).



**Figure 3.** Gene expression in human fibroblasts (HF) from normal skin, hypertrophic scar, and keloid scar is dependent on cell density and media composition. Quantitative real-time PCR was used to measure expression of (A) *COL1A1*, (B) *COL1A2*, (C) *MMP1*, (D) *MMP3*, (E) *PAI-1*, and (F) *PAI-2*. Expression levels were normalized to postconfluent normal fibroblasts in DFBS (dotted line = 1); mean normalized expression levels  $\pm$  SEM are shown. Note that in (D) and (F), expression is plotted using a logarithmic scale. Statistically significant differences ( $t$  test) are indicated.

As expected, *COL1A1* expression tended to be higher in keloid fibroblasts compared with normal fibroblasts (Figure 3A). In HFPM, expression of *COL1A1* was significantly higher in keloid fibroblasts compared with normal or HTS fibroblasts at all three cell densities examined. In DFBS, *COL1A1* was significantly higher in keloid fibroblasts compared with normal and HTS fibroblasts only when analyzed at subconfluent density. The greatest difference in *COL1A1* expression observed between normal and keloid fibroblasts, a 2.9-fold difference, was observed at confluence in HFPM.

Expression of *COL1A2* was significantly higher in fibroblasts cultured in DFBS compared with HFPM for all three cell strains at all cell densities examined (Figure 3B). As observed for *COL1A1*, *COL1A2* expression was also influenced by cell density: *COL1A2* expression was significantly higher in confluent fibroblasts compared with preconfluent fibroblasts for all three cell strains ( $P < .05$ ) and was significantly higher in HTS fibroblasts at postconfluent compared with confluent density ( $P < .05$ ). Differences in *COL1A2* expression between keloid and normal fibroblasts were only statistically significant at preconfluent and confluent densities in HFPM, and as with *COL1A1*, the greatest fold difference between normal and keloid cells (2.15-fold) was observed in confluent cells cultured in HFPM (Figure 3B).

Expression of *MMP1* was significantly impacted by genotype, media formulation, and cell density. Keloid fibroblasts exhibited significantly reduced *MMP1* expression compared with normal fibroblasts when cultured in either media type and examined at preconfluent and confluent densities (Figure 3C). The greatest fold difference in *MMP1* expression between normal and keloid fibroblasts, a 2.52-fold decrease, was observed in confluent cells in either medium formulation. HTS fibroblasts had significantly higher *MMP1* expression than keloid fibroblasts if cultured in HFPM and analyzed at confluent or postconfluent densities. If cells were cultured in DFBS compared with HFPM, *MMP1* expression was significantly reduced in preconfluent and confluent fibroblasts of all three cell strains (Figure 3C). In postconfluent cells, culture media had a significant impact on *MMP1* expression in HTS fibroblasts (Figure 3C) but not normal or keloid fibroblasts. The effects of cell density on *MMP1* expression were greatest when cells were cultured in HFPM and were dependent on cell genotype. Normal and HTS fibroblasts in HFPM showed a significant increase in *MMP1* expression between preconfluent and confluent cell densities ( $P < .001$ ), followed by a significant decrease in expression when cells were grown to postconfluence ( $P < .001$ ). A

trend toward decreasing *MMP1* expression with increasing cell density was observed in DFBS medium, but the difference was only statistically significant ( $P = .02$ ) for the comparison between HTS cells analyzed at preconfluent and confluent densities.

In contrast to *MMP1*, *MMP3* expression was significantly increased in fibroblasts cultured in DFBS compared with HFPM (Figure 3D). When comparing cells of the same genotype cultured to similar densities in the different media formulations, the fold differences ranged from 2.7-fold higher expression (normal fibroblasts at postconfluence) to 20-fold higher expression (normal or HTS fibroblasts at confluence) of *MMP3* in DFBS than HFPM. Keloid fibroblasts displayed significantly lower *MMP3* expression than normal fibroblasts in both media formulations and at all three cell densities. Expression of *MMP3* showed statistically significant decreases with increasing cell density; expression was lower in confluent compared with preconfluent cells, and in postconfluent vs confluent cells, for all three cell strains in both media formulations ( $P < .05$ ).

Expression of the serine protease inhibitor *PAI-1* was impacted by media formulation, and the response varied based on genotype and cell density. Expression of *PAI-1* in normal skin fibroblasts analyzed at preconfluent and confluent densities was significantly lower in DFBS than in HFPM, but at postconfluent densities, *PAI-1* was significantly higher in DFBS than in HFPM (Figure 3E). *PAI-1* expression in HTS fibroblasts was significantly lower in DFBS vs HFPM at preconfluent and confluent densities but not at postconfluent densities. In keloid fibroblasts, *PAI-1* was significantly lower in DFBS vs HFPM in confluent fibroblasts but was significantly higher in DFBS vs HFPM in postconfluent fibroblasts. Previous studies reported increased *PAI-1* expression in keloid fibroblasts compared with normal skin fibroblasts.<sup>28</sup> In this study, the magnitude and relative difference in *PAI-1* expression between normal and keloid fibroblasts was dependent on media composition and cell density. For example, keloid fibroblasts expressed significantly higher levels of *PAI-1* if cultured in HFPM or DFBS and analyzed at confluent or postconfluent densities (Figure 3E). However, in preconfluent keloid vs normal fibroblasts, significantly elevated expression was seen in DFBS but significantly decreased expression was seen in HFPM (Figure 3E).

*PAI-2* expression has not been previously characterized in abnormal scar-derived fibroblasts. In this study, *PAI-2* expression levels were found to be increased in scar-derived cells and were dependent on media compositions and cell densities (Figure 3F). At preconfluent density, *PAI-2* expression in normal fi-

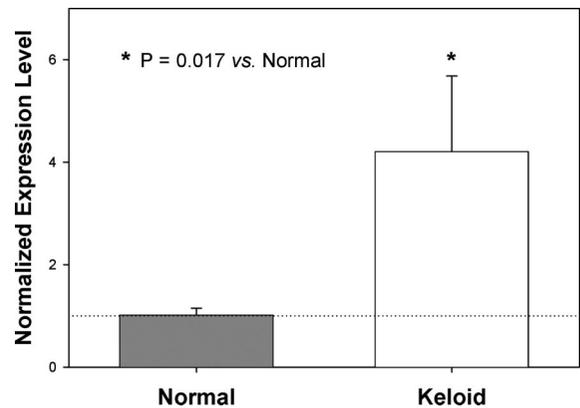
broblasts was not impacted by media formulation, but HTS and keloid fibroblasts expressed significantly lower levels of *PAI-2* in DFBS compared with HFPM. At confluence, all three cell strains expressed significantly lower levels of *PAI-2* in DFBS than in HFPM. Interestingly, when analyzed in postconfluent cells, expression of *PAI-2* was significantly higher in DFBS than in HFPM. For each cell density and in both media formulations, keloid fibroblasts expressed significantly higher levels of *PAI-2* than normal skin fibroblasts or HTS fibroblasts. The greatest difference in expression between normal and keloid fibroblasts, a 16.1-fold increase, was found in cells cultured to postconfluent density in HFPM. The media formulation used for cell culture affected the change in *PAI-2* gene expression observed as a function of cell density. For example, *PAI-2* expression significantly decreased in each strain as cell numbers increased from preconfluence to confluence and from confluence to postconfluence ( $P < .001$  for each comparison) when cultured in HFPM. However, when cultured in DFBS, significant changes in *PAI-2* expression were not observed as cell densities increased.

### *PAI-2* Expression in Normal and Keloid Fibroblasts

Because increased *PAI-2* expression has not been previously reported in abnormal scar fibroblasts, expression in 10 additional fibroblast strains was analyzed: 3 isolated from normal skin and 7 isolated from keloid scars (Table 1). Cells at passage 2 were cultured in HFPM and analyzed at confluence. Consistent with the data presented in Figure 3F, expression of *PAI-2* was significantly increased in keloid fibroblasts compared with normal skin fibroblasts (Figure 4).

## DISCUSSION

The purpose of this study was to examine the combined effects of culture media formulation and relative cell density on gene expression in cells derived from normal human skin or abnormal scar. Fibroblasts are commonly cultured using DMEM containing 10% bovine serum, which promotes adequate cell growth but may not provide the optimal environment for proliferation. Our results indicate that fibroblasts grown in HFPM, which has reduced serum (4%) but is supplemented with purified mitogens, display increased proliferation and a more homogenous phenotype than cells cultured in DFBS. After confluence, cells in HFPM continue to proliferate; in contrast, fibroblasts cultured post-confluence in DFBS display a heterogeneous phenotype with a high proportion of enlarged, flattened cells resembling postmitotic fibro-



**Figure 4.** *PAI-2* expression is increased in keloid-derived fibroblasts. Quantitative real-time PCR was used to measure expression of *PAI-2* in normal skin fibroblasts ( $n = 3$  cell strains) and keloid fibroblasts ( $n = 7$  cell strains); expression levels were normalized to the mean expression in normal fibroblasts (dotted line = 1). Shown are mean normalized expression levels  $\pm$  SEM. Statistical analysis was performed using the Mann-Whitney rank-sum test.

blasts.<sup>45</sup> These observations were similar for all three strains of fibroblasts examined, although there were overall differences between normal and scar-derived fibroblasts in cell numbers obtained in the different culture media at different time points.

Differential responses to changes in media formulation were observed between normal and scar-derived fibroblasts, but this was a gene-specific phenomenon that varied at different cell densities. For example, *PAI-1* expression was lower in each of the three fibroblast strains after cells were cultured in DFBS compared with HFPM and analyzed at preconfluent or confluent densities. However, normal and keloid fibroblasts expressed higher levels of *PAI-1* in DFBS compared with HFPM at postconfluent density. Similarly, preconfluent HTS and keloid fibroblasts had lower *PAI-2* expression when cultured in DFBS vs HFPM, but no significant change was observed in normal fibroblasts; however, postconfluent cells of each genotype had higher *PAI-2* expression in DFBS vs HFPM. At confluent densities, normal fibroblasts showed increased *PAI-2* in DFBS medium vs HFPM, whereas HTS and keloid fibroblasts had decreased *PAI-2* expression in DFBS. In contrast to the changes observed for *PAI-1* and *PAI-2*, *COL1A1* and *COL1A2* levels for all three fibroblast strains at all three densities were higher in DFBS than in HFPM. For *MMP1* and *MMP3*, expression was lower in DFBS than HFPM for all three cell strains at all three densities. These results imply differential regulation of specific genes by serum, growth factors, and cell-

cell contacts and suggest that the relevant regulatory mechanisms are impacted by the abnormal scar phenotype.

Although culture conditions were held constant, a limitation of this study is that only one strain of each scar type was examined in detail in the different media formulations and cell densities. However, the observed gene expression patterns are in general agreement with published data for expression of all genes examined in normal and scar-derived cells, except for *PAI-2*, which has not been previously examined in scar-derived fibroblasts. This suggests that the cell strains selected for analysis were representative of normal, HTS, and keloid fibroblasts.

HFBM was developed as an optimized medium for primary culture of human dermal fibroblasts intended for use in engineered skin substitutes for grafting in acute wounds.<sup>39</sup> This application requires rapid expansion of cells from relatively small biopsies to obtain large populations of proliferative cells for timely wound closure. Levels of bovine serum in HFBM are reduced, and factors that stimulate fibroblast proliferation have been added. These include EGF, insulin, hydrocortisone, and vitamin C (ascorbic acid-2-phosphate). EGF is a well-established mitogen for fibroblasts. Interestingly, Delany and Brinckerhoff<sup>46</sup> reported coordinately increased expression of *MMP1* and *MMP3* in fibroblasts cultured in DMEM containing 10% FBS + 10 ng/mL EGF.<sup>46</sup> In this study, *MMP1* was generally increased but *MMP3* expression was decreased in fibroblasts cultured in HFBM, which contains 10 ng/mL EGF. The difference in the results obtained may be due to the reduced levels of serum in HFBM compared with the previously published study or may be due to interactions with other factors present in the medium. Alternatively, differences in cell passage may be responsible for the disparate results, as this has been shown to impact gene expression<sup>23</sup>; passage 2 cells were used in this study, and Delany and Brinckerhoff<sup>46</sup> used cells between passages 3 and 11.

Insulin is mitogenic to fibroblasts in vitro and has been shown to stimulate collagen expression and accelerate burn wound healing.<sup>47-49</sup> In our study, fibroblasts cultured in HFBM, which contains 5  $\mu$ g/mL insulin in addition to 4% serum, had lower expression of *COL1A1* and *COL1A2* compared with cells cultured in 10% FBS. FBS is expected to contain small amounts of insulin, but the levels in DFBS should be substantially lower than in HFBM. The increase in collagen expression in DFBS may be due to other growth factors present in bovine serum, such as TGF- $\beta$ 1, which induce collagen expression in dermal fibroblasts.<sup>50</sup>

Ascorbic acid is an important cofactor for collagen biosynthesis and is a natural antioxidant that helps protect the body from harmful effects caused by reactive oxygen species. Oxidative stress, which occurs when the body has insufficient antioxidant capacity to protect against reactive oxygen species, is a feature of acute systemic inflammation that accompanies major trauma or burn injury.<sup>51,52</sup> Oxidative stress after burn injury is accompanied by decreased ascorbic acid levels, which impacts cardiovascular function and wound healing.<sup>51</sup> Consistent with increased collagen synthesis observed in fibroblasts in vitro, studies have demonstrated that vitamin C improves wound healing by enhancing collagen synthesis and fiber organization.<sup>53,54</sup> However, vitamin C-stimulated collagen production is counteracted by an inhibitory effect of hydrocortisone.<sup>38,55</sup> Although hydrocortisone was found to inhibit collagen synthesis in fibroblasts from normal tissue, collagen production in keloid fibroblasts was not significantly reduced.<sup>56</sup> This observation may explain in part why, in this study, greater differences in collagen expression between normal and keloid fibroblasts were observed in HFBM compared with DFBS. The clinical implications of reduced vitamin C after burn injury and vitamin C supplementation on development of abnormal scarring have not yet been explored.

The overexpression of *PAI-2* in keloid-derived fibroblasts suggests a role for *PAI-2* in development of keloid scarring. As a member of the serpin supergene family, it was generally assumed that the main function of *PAI-2* was inhibition of uPA, as is the case for *PAI-1*. However, studies have failed to localize *PAI-2* at the cell surface, where uPA is found; instead, most *PAI-2* is found within the cytosol, suggesting that it has other functions.<sup>57</sup> *PAI-2* is among a subset of genes highly induced in fibroblasts under mechanical stress, a condition that results in fibroblast activation. This has also been referred to as a “synthetic phenotype” because genes involved in connective tissue synthesis are stimulated, and matrix degrading genes are inhibited, in activated fibroblasts.<sup>58</sup> Mechanical tension has been proposed to play a role in HTS and keloid development, because these scars tend to occur in body sites where high skin tension is present.<sup>4</sup> *PAI-2* is also highly expressed in scleroderma, which is characterized by extensive skin fibrosis and an activated fibroblast phenotype.<sup>36</sup> A variety of functions and activities have been described for *PAI-2*, and these vary in different cellular contexts.<sup>59</sup> *PAI-2* overexpression in the epidermis of transgenic mice led to increased susceptibility to chemically induced papilloma formation, which was proposed to be due to its antiapoptotic effect.<sup>60</sup> This effect may be mediated by

PAI-2 binding to retinoblastoma protein (Rb), which regulates apoptosis and cell proliferation.<sup>61</sup> Hypothetically, increased PAI-2 in keloid fibroblasts may lead to increased fibrosis due to inhibition of apoptosis, although it may also be one reflection of an activated fibroblast phenotype.

In conclusion, the results of this study emphasize the critical role of culture conditions in the regulation of cell growth behavior, the interpretation of gene expression data, and for comparison of cells representing normal and fibrotic phenotypes. When comparing results obtained from different laboratories, differences in the methods used for establishing cultures, the formulation of culture media, the cell passage number, and the relative cell density at the time of analysis must all be taken into consideration, as these factors all impact the proliferative state of cells, which in turn effects gene expression profiles. The results of this study suggest that individual genes respond differently to changes in cell density and media formulation, even if these genes serve similar functions. Therefore, the use of gene expression analysis for identification of functionally important differences between normal and scar-derived cells must account for different responses to environmental factors, including culture conditions, as well as intrinsic factors, such as genotype.

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