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ORIGINAL RESEARCH REPORT

Paracrine Activity from Adipose-Derived Stem Cells on In Vitro Wound Healing in Human Tympanic Membrane Keratinocytes

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Stem cell therapies for tympanic membrane repair have shown initial experimental success using mesenchymal stem cells in rat models to promote healing; however, the mechanisms providing this benefit are not known. We investigated in vitro the paracrine effects of human adipose-derived stem cells (ADSCs) on wound healing mechanisms for human tympanic membrane-derived keratinocytes (hTM) and immortalized human keratinocytes (HaCaT). ADSC conditioned media (CM_{ADSC}) were assessed for paracrine activity on keratinocyte proliferation and migration, with hypoxic conditions for ADSC culture used to generate contrasting effects on cytokine gene expression. Keratinocytes cultured in CM_{ADSC} showed a significant increase in cell number compared to serum-free cultures and further significant increases in hypoxic CM_{ADSC}. Assessment of ADSC gene expression on a cytokine array showed a range of wound healing cytokines expressed and under stringent hypoxic and serum-free conditions was upregulated (*VEGF A, MMP9, Tissue Factor, PAI-1*) or downregulated (*CXCL5, CCL7, TNF-α*). Several of these may contribute to the activity of conditioned media on the keratinocytes with potential applications in TM perforation repair. VEGFA protein was confirmed by immunoassay to be increased in conditioned media. Together with gene regulation associated with hypoxia in ADSCs, this study has provided several strong leads for a stem cell–derived approach to TM wound healing.

Keywords: gene expression, growth factors, MSC, wound healing

Introduction

The skin is a protective barrier against the external environment and is routinely subjected to abrasive or penetrating injuries. Wound healing commences immediately after injury with an orchestrated activation of intracellular and intercellular pathways to reestablish tissue integrity and homeostasis [1]. The tympanic membrane is a specialized skin-like structure, which is protected from casual trauma by its anatomical location deep within the bony ear canal, but it remains subject to injury from air pressure, iatrogenic or accidental perforation by objects inserted into the ear canal, or most commonly as a consequence of otitis media. The tympanic membrane also has a robust injury response, but failure to heal occurs in up to 15% of patients, leaving a chronic perforation. The mechanisms of healing and failure-to-heal are of considerable interest, but relatively poorly defined. Unlike cutaneous wounds, which have a source of regenerative tissue at the base [2], TM perforations are full-thickness wounds and rely entirely on tissues at their lateral margin for regenerative responses. TM regeneration is very effective, with up to 90% of TM perforations healing spontaneously without intervention [3,4]. Keratinocytes lead the wound healing process by migrating across the perforation and proliferating to restore the epidermal layer, followed by the underlying granulation tissue and mucosal epithelial tissues to restore the middle and inner layers, respectively [5,6]. Where healing fails, chronic wounds may attract surgical treatment, but with increasing knowledge of TM biology it is envisaged that nonsurgical treatment with bioactive molecules may soon prove suitable in many cases [7], thus reducing cost and providing greater access to treatment outside of a surgical facility. A number of molecular entities have been tested as suitable treatments, but without a clear choice of factors emerging; research is ongoing in this area.

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One avenue relatively unexplored is stem cell stimulated wound healing. One of the few reports of stem cell healing for TM is the delivery of human mesenchymal stem cells (MSCs) into the middle ear, which promoted healing of acute and chronic perforations in rat ears [8]. Recent work showed that delivery of mouse bone marrow MSC on hyaluronic acid scaffold to perforated mouse tympanic membrane was able to promote healing of acute perforation [9] compared to scaffold alone. The molecular mechanism of stem cell mediated healing was however not discovered in either study. MSCs applied in other wound healing models have been shown to produce mediators that enhance wound healing locally through improved cell proliferation and viability [10,11], tissue remodeling by reepithelialization [8,12–16], angiogenesis [12,14,15,17,18], and inflammation [14,19], in a paracrine manner. These paracrine mediators may ultimately be able to reactivate the wound healing cascade in chronic wound healing [20,21].

We propose that paracrine mechanisms might prove useful to promote wound healing in TM perforations and explain some of the wound healing activity derived from MSC in rats. In the present study, we evaluated in vitro models for paracrine activity from human adipose-derived MSCs on human tympanic membrane keratinocytes and the HaCaT keratinocyte cell line. Hypoxic conditioning of the MSC was an effective stimulator of proliferative and chemotactic activity. We further studied the effect of hypoxic conditioning on the MSC wound healing transcriptome and identified several candidate factors for future study.

Materials and Methods

Culture of primary hTM keratinocytes and HaCaT cells

Primary hTM keratinocytes derived from normal human tympanic membrane explants were used as previously described [22]. The St John of God HealthCare Ethics Committee approved collection of excess tympanic membrane tissue from patients undergoing otological procedures at St John of God Hospital (Subiaco, Australia). The HaCaT cell line was supplied by the Burns Injury Research Unit, School of Surgery, University of Western Australia. Both cell types were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L D-glucose (Gibco), supplemented with 10% fetal bovine serum (FBS; Bovogen), 1% Penicillin-Streptomycin (Gibco), and incubated in a humidified cell culture incubator at 37°C, with 5% carbon dioxide (CO_2) until confluent. Cells were passaged every 3–4 days.

Culture of adipose-derived stem cell

Human adipose-derived stem cells (ADSCs; Lonza) were cultured in DMEM containing 1 g/L D-glucose supplemented with 10% FBS and 1% Penicillin-Streptomycin, with spent medium replaced every 2–3 days.

Collection of ADSC-conditioned medium

ADSCs were cultured to 80%–90% confluence in 75 cm^2 tissue culture flasks, rinsed with 1 × phosphate-buffered saline (PBS), pH 7.2 (Gibco), and incubated with 10 mL of serum-free (S−) DMEM (1 g/L D-glucose) for 8 h before replacement with fresh serum-free medium. Two flasks were incubated under standard conditions (37°C, 5% CO_2) and two under hypoxic conditions (GENbox Jar; BioMérieux) created by an anaerobic atmosphere generator sachet (BioMérieux) at 37°C. An indicator strip (BioMérieux) was included in the sealed chamber to confirm gas composition (<0.1% O_2 and 15% CO_2). All four flasks were incubated for 48 h. Conditioned media were then collected using a sterile 10 mL syringe (BD) and filtered through a 0.2 μm filter membrane (PALL) to remove cell debris. The media collected are referred to as Nx ADSC conditioned media (CM_{ADSC}) and Hx CM_{ADSC}, respectively.

Total RNA isolation and complementary DNA synthesis

Total RNA was extracted from ADSCs and hTM keratinocytes according to manufacturer's instructions (FavorPrep™ Tissue Total RNA Mini Kit; Favorgen Biotech Corp), then RNA concentration and purity were measured by spectrophotometry (Epoch Take3; BioTek Instruments, Inc.). Synthesis of complementary DNA (cDNA) was then performed (RT^2 First Strand Kit; Qiagen) according to manufacturer's instructions with 0.5 μg of RNA.

Real-time polymerase chain reaction of human wound healing array

Human Wound Healing polymerase chain reaction (PCR) array was performed according to manufacturer's instruction (RT^2 Profiler; Qiagen) on the cDNA of $ADSC_{Hx}$, $ADSC_{Nx}$, and hTM keratinocyte S−. Briefly, cDNA, SYBR green master mix, and water were combined to a final volume of 2,700 μL. A volume of 25 μL per well was added to the 96-well array plate and run on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad) under the following conditions: 95°C for 10 min to activate HotStart DNA Taq Polymerase and 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 1 min. Threshold cycles (C_t) were determined using the exponential growth phase and the baseline signal from fluorescence versus cycle number plots. Relative messenger RNA (mRNA) expression levels were determined using the Livak method ($2^{-\Delta\Delta Ct}$) with beta-2-microglobulin (B2M) selected as the most stable housekeeping gene. Each experiment was performed in duplicate array plates repeated for all $ADSC_{Hx}$, $ADSC_{Nx}$, and hTM keratinocyte S^-.

Gene ontology analysis of hypoxia-induced gene expression changes in cultured ADSC

Gene ontology analysis was performed using the g:Profiler platform for enrichment analyses to identify biological functions affected by hypoxic conditions in cultured ADSC (http://biit.cs.ut.ee/gprofiler/welcome.cgi) [23]. Query lists of gene coding for secreted proteins of potential paracrine effect on keratinocytes were uploaded and analyzed for up- and downregulated genes in separate analyses. Benjamini–Hochberg false discovery rate (FDR) was chosen as the statistical method for multiple testing correction.

Total protein assay

Total protein concentration in both Hx and Nx CM_{ADSC} were quantified using a Bradford Protein Assay (Quick Start;

Bio-Rad) according to manufacturer's protocol. Bovine Serum Albumin standards were used over a range of 1.25–10 μg/mL. After incubating the CM_{ADSC} with dye reagent, the absorbance was measured at 595 nm using a spectrophotometer (Epoch; BioTek Instruments, Inc.) and protein concentrations of the CM_{ADSC} read from the standard curve.

Quantification of specific protein levels

Secreted VEGF-A, IL6, IL1B, FGF2, HB-EGF, and FGF7 proteins in CM_{ADSC} were quantified using enzyme-linked immunosorbent assay (ELISA, Quantikine kits; R&D Systems) according to the manufacturer's protocol. Briefly, protein standard curves were prepared by serial dilution. In duplicates, samples were loaded into microplate wells precoated with monoclonal antibodies. After incubation for 2–3 h, wells were washed to remove unbound proteins and then enzyme-linked polyclonal antibody was added to the wells and incubated. Final washing was done to remove unbound antibody-enzyme reagent, then substrate containing hydrogen peroxide and tetramethylbenzidine was added and incubated for 30 min before stopping the reaction by acidification. Optical density was measured at 450 nm on the spectrophotometer with a wavelength correction at 570 nm. Secreted protein concentrations were expressed in ng per mg of total protein, calculated from either linear standard curves or four parameter logistic (4-PL) curve fit by interpolating sample values.

MTS assay for proliferation

Cell proliferation was analyzed by colorimetric assay (CellTiter 96® AQ_{ueous} One; Promega Corporation). Briefly, keratinocytes were serum-starved for 8 h before experimentation, resuspended in test medium [5,000–10,000 cells in 10% serum (S+), serum free (S–), or 50:50 mix of conditioned media and S– media], and seeded in 96-well plates for a 24-h or 48-h incubation at 37°C, 5% CO_2. Following incubation, 20 μL of colorimetric solution was added to each well, and plates were incubated for 2 h. Absorbance was measured at 490 nm wavelength using the Epoch colorimetric plate reader (BioTek Instruments, Inc.). Samples were read in triplicate, averaged, and corrected for background optical density. The effect on proliferation was expressed as a fraction of the S– control treatment for each cell, respectively.

Scratch test assay for migration

A scratch wound assay was performed on hTM keratinocytes and HaCaT cells to assess cell migration when cultured in Hx CM_{ADSC}, Nx CM_{ADSC}, and S^+. Initially, 500,000 keratinocytes were seeded in 12-well culture plates with S+ media and grown to confluence. Following 8 h preincubation in S– medium, cell monolayers were wounded with a sterile 200 μL pipette tip with light vacuum applied to clear debris from the wound. Cells were then washed with PBS and incubated with the corresponding test medium. Microscopic images were taken immediately after scratch wounding (t_0) and after 10 h on an Olympus BX60 microscope with a DP70 digital camera (Olympus). Wound gaps were measured using ImageJ (1.48v) software. Each gap was measured 45 times. All measurements were repeated in triplicate and averaged. The migration of keratinocytes into the gap was calculated as:

$$\%\,\text{Gap closure} = [(\text{Gap length}_{t\,=\,10\,h} - \text{Gap length}_{t\,=\,0\,h})/\text{Gap length}_{t\,=\,0\,h}] \times 100\%$$

The results are expressed as a fraction of the S– control treatment as above.

Statistics

Quantitative data are presented as mean ± standard error of the mean (SEM). Microsoft Excel was used for all data analysis. Statistical methods used include:

- One-way analysis of variance (ANOVA) for Proliferation and Migration experiments
- Dunnett's test as a post hoc test for individual treatment group comparisons against S– control group for both Proliferation and Migration experiments
- Student *t*-test for ELISA and PCR data analysis

Statistical level of confidence was set at $P < 0.05$ unless otherwise stated.

Results

Cell proliferation and migration assays were performed using Hx and Nx CM_{ADSC} on hTM and HaCaT keratinocytes with serum-containing (S+) and serum-free (S–) media as positive and negative controls. Figure 1 shows that ADSC cultures were viable before and after conditioning in hypoxic or normoxic conditions.

Effect of CM_{ADSC} on keratinocyte proliferation

Representative images of proliferation responses in hTM and HaCaT keratinocytes over 48 h are shown in Fig. 2. In hTM keratinocyte cultures, serum produced a

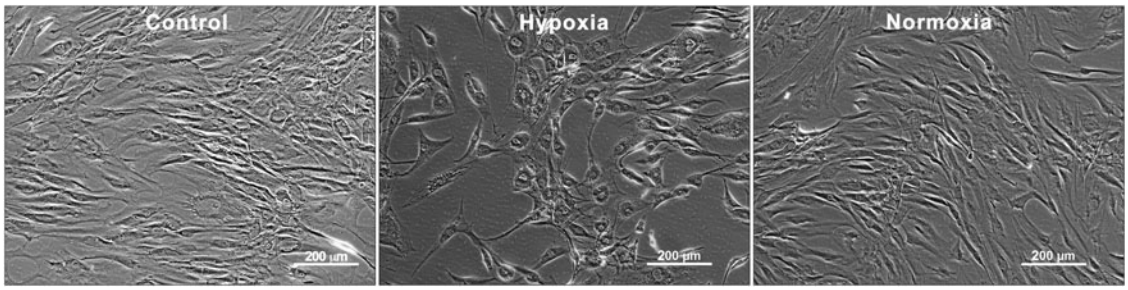


FIG. 1. Human ADSC before and after hypoxic and normoxic conditioning for 48 h. Scale bars = 200 μm. ADSC, adipose-derived stem cell.

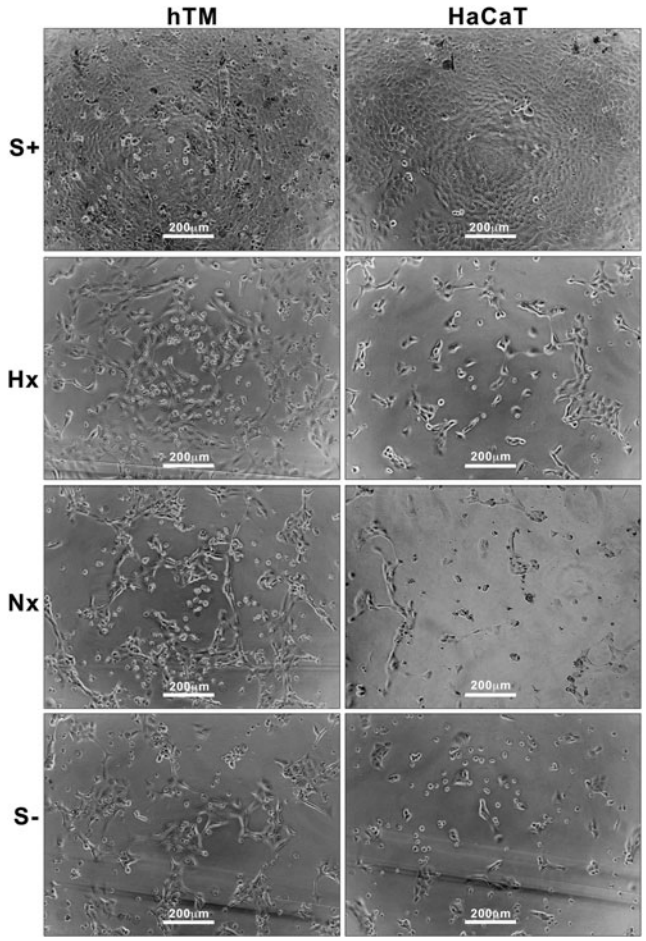


FIG. 2. *Left columns*: hTMt in test medium (S+, Hx and Nx CM_{ADSC}, S−) during proliferation test at 48 h; *right columns*: HaCaTs in test medium during proliferation test at 48 h. Scale bars = 200 μm. CM_{ADSC}, ADSC conditioned media.

substantial proliferative response at 24 and 48 h compared to serum-free cultures (Fig. 3). Hx CM_{ADSC} media produced a significantly greater proliferative response ($P < 0.05$) than serum-free media and quantitatively as much as 83% and 95% of the serum response at 24 and 48 h, respectively. Nx CM_{ADSC} however only showed significant ($P < 0.05$) proliferative response at 48 h, up to 79% of the serum response. When comparing Hx and Nx CM_{ADSC}, there was a significant difference ($P < 0.05$) between the proliferative effects on hTM keratinocytes at both 24 and 48 h.

In HaCaT keratinocytes, the proliferative effect of serum was significant ($P < 0.05$) at 24 h, but not at 48 h compared to serum-free controls (Fig. 3). Proliferative responses of HaCaT keratinocytes to Hx CM_{ADSC} was significant at both 24 and 48 h, quantitated at 106% and 109% of serum response, respectively. Nx CM_{ADSC} treatments were not significant at 24 h, but at 48 h responses were significantly greater than serum-free controls of up to 9% compared to serum-free controls. Both CM_{ADSC} treatments produced a proliferative response larger than the serum treatment effect at 48 h. When comparing Hx and Nx CM_{ADSC}, there was a significant difference ($P < 0.05$) between the proliferative effects on HaCaT at both 24 and 48 h.

Effect of CM_{ADSC} on keratinocyte migration

Migration assays were performed on hTM and HaCaT keratinocytes over 10 h to compare the effect of Hx and Nx CM_{ADSC} measured against serum-free control media (Figs. 4 and 5). Representative images of wound gaps in all test media are presented in Fig. 4, and the quantitative data are presented as the normalized percentage gap closure in Fig. 5.

Hx CM_{ADSC} and Nx CM_{ADSC} produced a significantly greater ($P < 0.05$) gap closure effect on hTM keratinocytes compared to S− media controls. Hx CM_{ADSC} effects on hTM keratinocytes were significantly higher than Nx CM_{ADSC} ($P < 0.05$). Gap closures were on average 27% and 10%, respectively, greater than in the absence of paracrine activity. In these experiments, hTM keratinocytes were found to maintain a migration capacity in the absence of serum ($P < 0.05$).

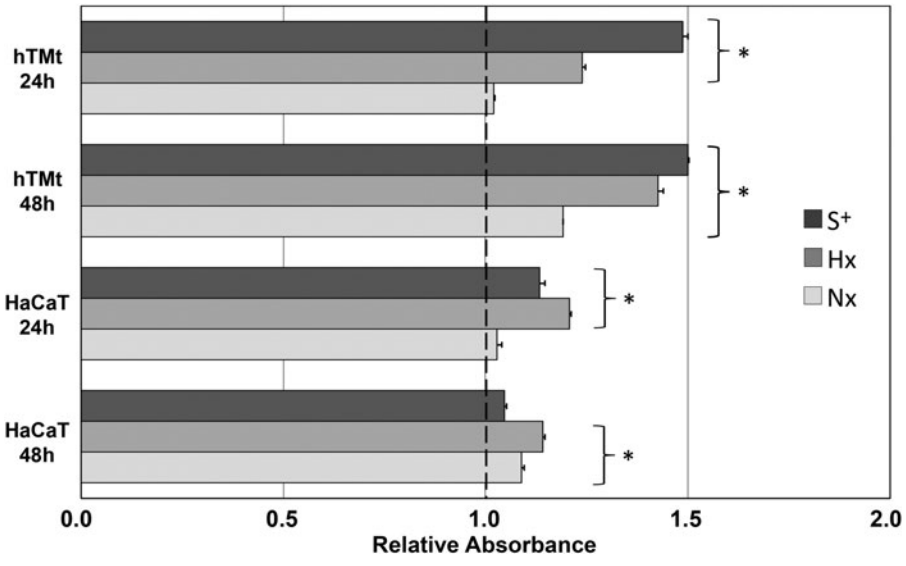


FIG. 3. Effect of S+, Hx and Nx, CM_{ADSC} on the proliferation of hTM and HaCaT keratinocytes over 24 and 48 h. Graph shows representative data of relative absorbance values normalized against the negative (S−) control. hTM keratinocytes and HaCaT keratinocytes had a greater proliferative response to Hx CM_{ADSC} than Nx CM_{ADSC}. Test media marked with *asterisk* (*) were significantly different to S− media ($P < 0.05$).

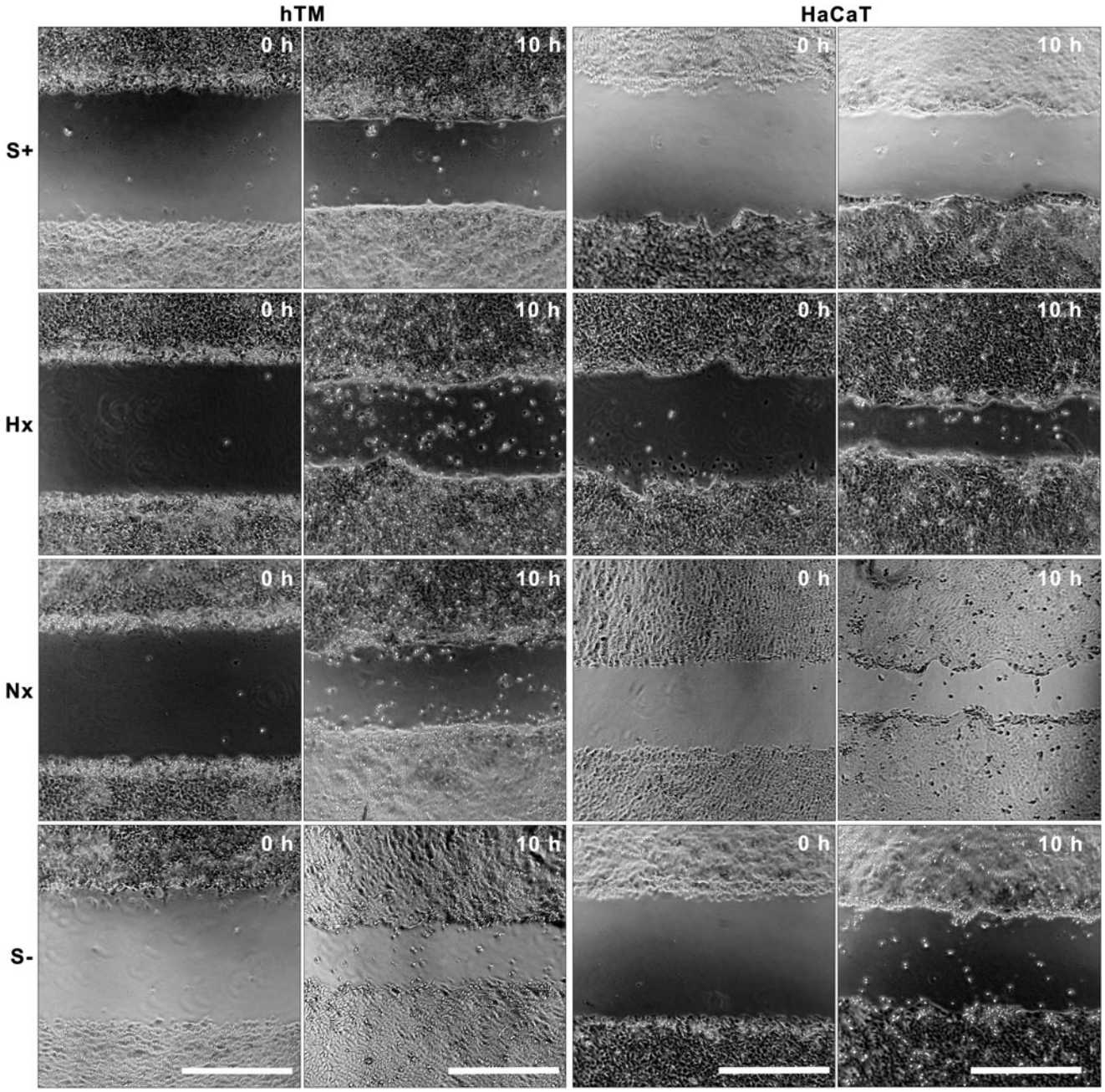


FIG. 4. Representative image of Migration test over 10 h; *left two columns*: hTM keratinocyte wound gaps in various test medium; *right two columns*: HaCaT keratinocyte wound gaps in various test medium. Scale bars = 500 μm.

In HaCaT keratinocytes, significant differences in migration were observed for both Hx and Nx CM_{ADSC} ($P < 0.05$). Gap closures were on average 37% in serum controls, 98% in Hx CM_{ADSC} and 43% in Nx CM_{ADSC} greater than in the S− media controls. HaCaT cells cultured in S− media migrated less well and maintained a 35% gap even after 28 h, while all other test wells were closed at this time.

Molecular identification of paracrine activity

PCR array for wound healing gene expression in ADSCs and hTM keratinocytes. PCR Wound Healing arrays were used to determine which of the 84 genes present on the array were expressed in ADSCs, as well as to perform a quantitative comparison of the effects of hypoxic and normoxic culture conditions. B2M was a stable housekeeping gene for these arrays, so was used to normalize all values, and C_t thresholds were manually set to the same value across all samples.

Gene expression. Among the 84 genes present on the array (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/scd), 67 were expressed in ADSCs and 17 genes were not detected (C_t values >35). Among the 17 genes not detected were some prominent regulators of keratinocyte growth, which could then be excluded as candidates for paracrine activity from ADSCs

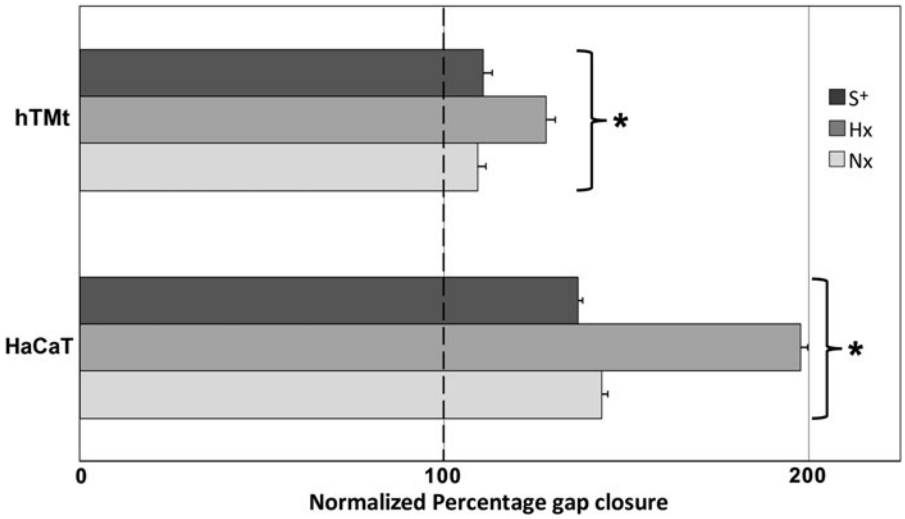


FIG. 5. Effect of S+, Hx and Nx, CM_{ADSC} on the migration of hTM and HaCaT keratinocytes over 10 h. Migration data over 10 h are represented as normalized percentage gap closure of negative control (S−) wells (at 100%). All test media marked with an *asterisk* (*) were significantly different ($P < 0.05$) from the corresponding S− controls.

on keratinocytes: EGF, TGFα, HGF, FGF10 (KGF2), IFNγ, and IGF-1 (Table 1).

Expression of wound healing genes in hTM keratinocytes was also examined in the same manner, and a largely similar set was found (Table 2), but with notable differences being absence of gene expression in keratinocytes for cell adhesion molecule integrin β3, collagens alpha 1A2, 4A1, 4A3, 5A1, 5A3, and 14A1, WISP1 and WNT5A, and presence of gene expression for integrin β6, TGFα, E-cadherin, MMP7, and TNF.

Hypoxia in ADSCs. Thirty-eight expressed genes were upregulated (2- to 62-fold) by hypoxia in ADSCs, 12 genes were downregulated (2- to 5-fold), and 17 were expressed, but showed less than twofold difference between the culture conditions. Taking into consideration that we were looking for secreted proteins, genes encoding for nonsecreted proteins were removed from the list of comparison between Hx and Nx ADSCs (Table 1). Genes not regulated by hypoxia, hence not likely related to the hypoxia-induced growth responses, included prominent keratinocyte growth regulators HB-EGF and FGF7. FGF2 was only 1.9-fold higher in Hx. Genes reduced by hypoxia were chemokines and cytokines, including prominently the keratinocyte growth factor TNFα.

Gene expression regulated by hypoxia. Tissue Factor (F3), showed the highest upregulation in this assay with more than 60-fold higher expression in Hx ADSCs than Nx ADSCs. Out of 56 genes coding for secreted proteins, 24 were upregulated by at least twofold; 13 showed similar expression levels; 7 were downregulated between two- to sixfold; and 12 genes did not meet the threshold of detection (C_t values >35 or blank) in the array.

Gene ontology analysis. The results are summarized in Table 3. For a list of regulated genes and all significant gene ontology terms, see Supplementary Figure S1. The genes upregulated by hypoxic conditions are rich in collagens IV and V particularly and, thus, related to basement membrane and fibrillar collagen formation in the extracellular matrix. Among the downregulated genes are multiple chemokine (C-X-C) ligands, showing that ADSC influenced by hypoxia may have a direct impact on the chemotaxis of leukocytes.

Protein concentration in conditioned media. To determine whether the hypoxia-mediated increase in gene expression of VEGFA, IL6, and IL-1B from the PCR array results was translated into increased protein secretion, CM_{ADSC} were analyzed using ELISA. FGF2, HB-EGF, and FGF7 were included in the analysis due to their potential involvement in TM wound healing mechanism. VEGFA, IL6, IL-1B, FGF2, and FGF7 were all observed at detectable levels in CM_{ADSC}. HB-EGF was not detected. Figure 6 shows the relative fold change in specific protein levels in Hx CM_{ADSC} against Nx CM_{ADSC}. VEGFA protein level was sixfold higher in Hx CM_{ADSC} than in Nx CM_{ADSC} ($P < 0.05$), while IL6 and IL-1B were not significantly higher ($P > 0.05$).

Discussion

The major finding of this study was that ADSCs produced a paracrine activity to promote wound healing mechanisms in hTM and HaCaT keratinocytes in vitro. Conditioned media from hypoxic ADSC cultures promoted further increase in both proliferation and migration of keratinocyte cell lines compared to normoxic conditioned media. To find active candidates for this paracrine effect, we identified which wound healing genes coding for secreted proteins were expressed by ADSC and were regulated by hypoxic culture conditions. We were also able to exclude some candidates based on a lack of gene expression, or no change with hypoxia. This study is the first to show that ADSC-secreted VEGF might influence keratinocyte growth and wound healing in a paracrine manner and could influence MSC therapy at the tympanic membrane.

ADSCs in TM wound healing and keratinocytes

Recent studies show that human bone marrow-derived MSCs promote healing of chronic TM perforation in rats [8] and that mouse bone marrow-derived MSCs promote healing of acute TM perforation in mice [9]. Neither study identified the mechanism of these effects. In this study, we show for the first time an improved in vitro wound healing of hTM keratinocytes using adipose-derived MSC paracrine

Table 1. Wound Healing Array Gene Expression of Adipose-Derived Stem Cells Regulated by Hypoxic Environment

Gene	P	*Fold Δ*	*Secreted*	*Gene*	P	*Fold Δ*	*Secreted*
F3	**0.028**	**61.49**	**N**	*MAPK3*	0.317	1.92	Y
MMP9	0.089	21.55	Y	*FGF2*	0.144	1.90	Y
VEGFA	**0.048**	**15.06**	**Y**	*CTSV*	0.281	1.79	N
WISP1	0.105	13.35	Y	*COL3A1*	0.229	1.78	Y
TAGLN	0.094	11.48	N	***FGF7***	**0.018**	**1.77**	**Y**
SERPINE1	0.112	11.29	Y	*RAC1*	0.396	1.66	N
COL4A3	**0.036**	**9.01**	**Y**	*RHOA*	0.421	1.58	N
IL1B	**0.002**	**8.11**	**Y**	*MMP2*	0.376	1.55	Y
MIF	0.098	7.46	Y	*ITGA6*	0.409	1.52	N
PLAT	0.120	7.02	Y	*PLAU*	0.428	1.47	Y
CTGF	**0.047**	**6.44**	**Y**	*MAPK1*	0.442	1.47	Y
COL5A1	**0.022**	**5.80**	**Y**	*COL1A2*	0.437	1.41	Y
COL4A1	**0.024**	**5.47**	**Y**	*COL1A1*	0.416	1.38	Y
IL6	**0.006**	**5.30**	**Y**	*ITGAV*	0.472	1.27	N
TIMP1	0.127	4.91	Y	*WNT5A*	0.451	1.12	Y
COL5A3	**0.047**	**4.34**	**Y**	*COL14A1*	0.156	1.05	Y
PTGS2	0.136	3.98	Y	***HBEGF***	**0.036**	**1.04**	**Y**
EGFR	**0.007**	**3.87**	**N**	*ITGA3*	0.350	−1.06	N
ITGA2	0.160	3.63	N	*ITGA4*	0.268	−1.08	N
ITGA1	**0.001**	**3.33**	**N**	*CSF3*	0.235	−1.09	Y
COL5A2	0.080	3.25	Y	*TGFBR3*	0.249	−1.15	N
CSF2	**0.027**	**3.12**	**Y**	*PDGFA*	0.236	−1.30	Y
PLAUR	0.140	3.11	Y	***CTSK***	**0.049**	**−2.10**	**Y**
ANGPT1	0.123	3.10	Y	*CXCL2*	0.087	−2.51	Y
TGFB1	0.186	3.00	Y	***CCL2***	**0.008**	**−2.67**	**Y**
ITGB5	0.210	2.99	N	*TNF*	0.153	−4.05	Y
ITGB1	0.252	2.90	N	*CCL7*	0.062	−4.73	Y
MMP1	0.260	2.70	Y	***CXCL1***	**0.033**	**−5.24**	**Y**
ITGA5	0.184	2.68	N	***CXCL5***	**0.023**	**−5.47**	**Y**
PTEN	0.218	2.47	N	*ACTC1*			N
ACTA2	0.062	2.39	N	*IL4*			Y
IL6ST	**0.005**	**2.23**	**N**	*IL10*			Y
PLG	0.273	2.22	Y	*CDH1*			N
CXCL11	0.211	2.22	Y	*TGFA*			Y
VTN	0.355	2.17	Y	*IGF1*			Y
ITGB3	0.284	2.14	N	*CD40LG*			N
STAT3	0.263	2.11	N	*CTSG*			N
CTNNB1	0.173	2.09	N	*EGF*			Y
B2M	-	1.00		*F13A1*			Y
				FGA			Y
				FGF10			Y
				IFNG			N
				IL2			Y
				ITGB6			Y

B2M, beta-2-microglobulin.
Increase fold Δ >2-folds
Not regulated by hypoxia
Decrease fold Δ <2-folds
Housekeeping Gene
Not expressed
Bold text shows $P < 0.05$.

Table 2. Wound Healing Gene Expression Profile of Hx Adipose-Derived Stem Cell, Nx ADSC, and hTM Keratinocytes

Gene	*Hx*	*Nx*	*hTMt*	*Gene*	*Hx*	*Nx*	*hTMt*	*Gene*	*Hx*	*Nx*	*hTMt*
ACTA2	+	+	+	*EGFR*	+	+	+	***MAPK1***	+	+	+
ACTC1	–	–	–	***F13A1***	–	–	–	***MAPK3***	+	+	+
ANGPT1	+	+	+	*F3*	+	+	+	***MIF***	+	+	+
CCL2	+	+	+	***FGA***	–	–	–	***MMP1***	+	+	+
CCL7	+	+	+	***FGF10***	–	–	–	***MMP2***	+	+	+
CD40LG	–	–	–	***FGF2***	+	+	+	***MMP7***	–	–	+
CDH1	–	–	+	***FGF7***	+	+	+	***MMP9***	+	+	+
COL14A1	+	+	_	***HBEGF***	+	+	+	***PDGFA***	+	+	+
COL1A1	+	+	+	***HGF***	–	–	–	***PLAT***	+	+	+
COL1A2	+	+	_	***IFNG***	–	–	–	***PLAU***	+	+	+
COL3A1	+	+	+	***IGF1***	–	–	–	***PLAUR***	+	+	+
COL4A1	+	+	_	***IL10***	–	–	–	***PLG***	+	+	+
COL4A3	+	–	_	***IL1B***	+	+	+	*PTEN*	+	+	+
COL5A1	+	+	_	***IL2***	–	–	–	***PTGS2***	+	+	+
COL5A2	+	+	+	***IL4***	–	–	–	*RAC1*	+	+	+
COL5A3	+	+	_	***IL6***	+	+	+	*RHOA*	+	+	+
CSF2	+	+	+	*IL6ST*	+	+	+	***SERPINE1***	+	+	+
CSF3	+	+	+	*ITGA1*	+	+	+	*STAT3*	+	+	+
CTGF	+	+	+	*ITGA2*	+	+	+	*TAGLN*	+	+	+
CTNNB1	+	+	+	*ITGA3*	+	+	+	***TGFA***	–	–	+
CTSG	–	–	–	*ITGA4*	+	+	+	***TGFB1***	+	+	+
CTSK	+	+	+	*ITGA5*	+	+	+	*TGFBR3*	+	+	+
CTSV	+	+	+	*ITGA6*	+	+	+	***TIMP1***	+	+	+
CXCL1	+	+	+	*ITGAV*	+	+	+	***TNF***	–	+	+
CXCL11	+	+	+	*ITGB1*	+	+	+	***VEGFA***	+	+	+
CXCL2	+	+	+	*ITGB3*	+	+	_	***VTN***	+	+	+
CXCL5	+	+	+	*ITGB5*	+	+	+	***WISP1***	+	+	–
EGF	–	–	–	*ITGB6*	–	–	+	***WNT5A***	+	+	–

Hx ADSC, Nx ADSC, and hTM keratinocytes expressed (+) or not expressed (–) where *highlighted boxes* indicate variations and *bold* represents secreted genes.

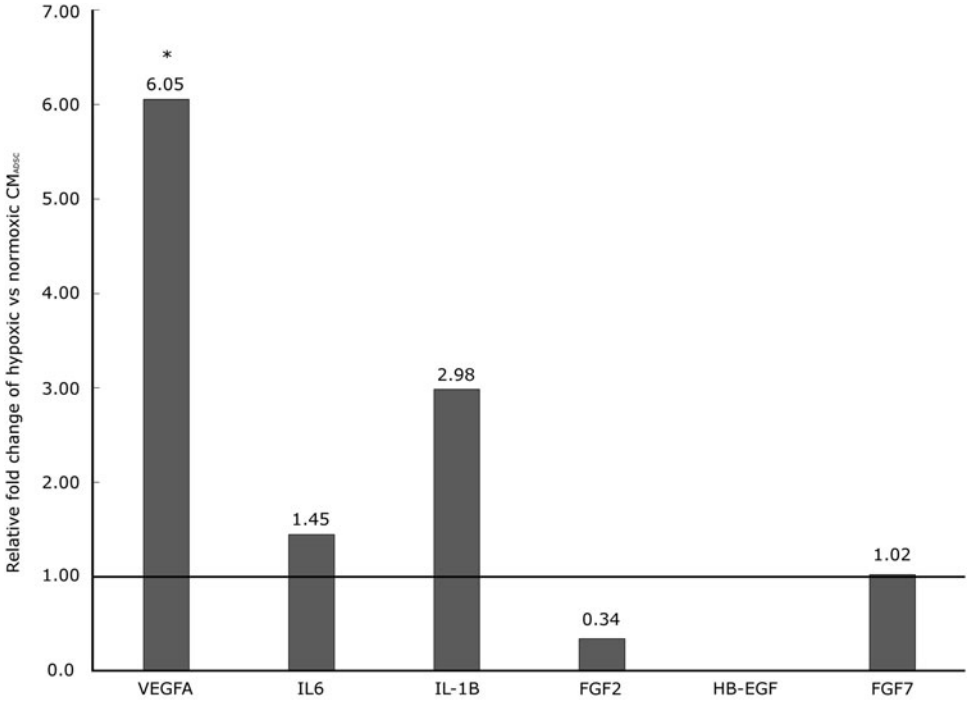
ADSC, adipose-derived stem cell.

Table 3. Gene Ontology Analyses of Up- and Downregulated Genes of Secreted Proteins in Adipose-Derived Stem Cell During Hypoxic Culture Conditions

Term type	*Term name*	P
Upregulated genes		
BP	Extracellular matrix disassembly	0.0456
BP	Negative regulation of cellular protein metabolic process	0.0273
BP	Regulation of proteolysis	0.0192
CC	Complex of collagen trimers	0.0133
CC	Intracellular	0.0406
CC	Endoplasmic reticulum lumen	0.0117
CC	Collagen type V trimer	0.0234
CC	Collagen trimer	0.0276
MF	Ion binding	0.0500
MF	Integrin binding	0.0094
Downregulated genes		
BP	Neutrophil chemotaxis	0.0005
BP	G-protein coupled receptor signaling pathway	0.0001
BP	Regulation of leukocyte migration	0.0001
BP	Chemokine-mediated signaling pathway	0.0001
BP	Regulation of leukocyte chemotaxis	0.0039
BP	Positive regulation of immune system process	0.0034
BP	Response to lipopolysaccharide	0.0071
MF	Chemokine receptor binding	0.0001

Organism: *Homo sapiens*; query list of significantly up- (19) and downregulated (7) genes; options: significant only, ordered query, no electronic gene ontology annotations, gene list as a statistical background: all genes in the RT^2 Profiler PCR array that were expressed by ADSC (69 genes); hierarchical filtering: best per parent; gene ontology analyses; significance threshold: Benjamini–Hochberg FDR corrected *P* value ($P < 0.0005$).

BP, biological process; CC, cellular component; FDR, false discovery rate; MF, molecular function; PCR, polymerase chain reaction.



	ng VEGFA per mg protein	ng IL6 per mg protein	ng IL-1B per mg protein	ng FGF2 per mg protein	ng HB-EGF per mg protein	ng FGF7 per mg protein
hADSC Hx	102.80	57.46	0.02	0.87	0.00	2.72
hADSC Nx	16.99	39.75	0.01	2.53	0.00	2.66
t-test	0.008	0.203	0.150	0.257	N/A	0.486

FIG. 6. Relative fold change of secreted VEGFA, IL6, IL-1B, FGF2, HB-EGF, and FGF7 in hypoxic and normoxic conditioned media after 48 h culture of ADSC (*$P \leq 0.05$).

activity. These data provide a plausible mechanism for the in vivo findings in rodents and support further investigation into the therapeutic potential of conditioned media or growth factor therapy, rather than the logistically more difficult cell implantation therapy.

Based on the wound-healing literature, keratinocyte proliferation and migration can be regulated by exposure to bioactive molecules from exogenous, paracrine, or autocrine sources. Factors involved include EGF, TGFα, TGFβ1, HB-EGF, FGF2, KGF, IL6, and VEGF A. [16,24–27]. In TM perforations, however, the molecular mechanisms regulating wound healing are less well studied [5,8,28], and the search for an optimal growth promoting activity is ongoing.

Response to normoxic and hypoxic CM_{ADSC}

In the present study, ADSCs in a normoxic environment enhanced proliferation and migration in keratinocytes consistent with previous studies on epithelial cells such as skin keratinocytes [29–32] or endothelial cells [32–34] and on nonepithelial cells like fibroblasts [16,33]. Conditioned media from hypoxic culture conditions produced a greater regenerative response in hTM and HaCaT keratinocytes, suggesting that in hypoxia ADSCs secreted more or different paracrine factors to that in normoxia. A hypoxic environment stimulates greater production of some paracrine factors from MSC [34] through activation of hypoxia inducible factor alpha (HIF-1α) transcription factor [2,35–38]. MSCs using glycolysis rather than mitochondrial respiration in hypoxic environments [39] increase their expression of paracrine factors [40–42]. The migration response in both hTM and HaCaT keratinocytes to Hx CM_{ADSC} was greater than responses to serum, indicating a high degree of responsiveness and specificity for chemotactic mechanisms by the paracrine mediators.

Identity of the paracrine activity from ADSCs

From a wound healing PCR array on ADSCs we identified gene expression of secreted paracrine factors and found genes expressed differently in Hx ADSCs versus Nx ADSCs. Eleven genes expressed but not regulated by hypoxia were set aside as not likely to explain the changes in paracrine activity seen in hypoxia. Among the genes for secreted proteins, 12 were not expressed, 24 were upregulated, and 9 were downregulated in Hx ADSCs. These are all candidates worthy of further consideration, as the differential expression in genes could be associated with the difference in paracrine activity secreted. (Table 4).

Several important keratinocyte growth regulators from the literature such as TGFα [24] and EGF were not expressed by ADSC. HB-EGF, another important regulator of TM wound healing [43,44], was expressed but not regulated significantly by hypoxia, and secreted protein was not detected. FGF-2, also used to promote TM wound healing in other studies [43,45–47], was expressed and secreted into the CM_{ADSC}, but was not regulated by hypoxia in our study. These data suggest that there is significant growth activity from factors other than

Table 4. Selected Genes Expressed by Adipose-Derived Stem Cells in This Study Categorized Based on Significant Relationship to Keratinocyte Growth

Gene	*Known relation to keratinocytes*	*Expression in ADSCs*
Chemotactic		
MMP9	Stimulates epithelial cell migration [55–57] MMP9-deficient mouse model showed arrested epithelial wound healing and mice were unable to remove fibrinogen matrix during wound healing [58]	Upregulated in Hx
CXCL1, 2, 5, 7, CCL2, 7	No known relation	Downregulated in Hx
Angiogenic		
VEGFA	Binds to VEGFR1 and VEGFR2 Keratinocytes express VEGFR1 [59,60] Keratinocytes also express and secrete VEGFA [59,61–63] Suggested proliferative signals in promoting repair through autocrine pathway in keratinocytes [60]	Upregulated in Hx
SERPINE1	One of the upregulated genes measured in HaCaT when exposed to serum [64] TGFB1 Found to increase expression of SERPINE1 in keratinocytes [65] Contributes to wound repair through cellular migration [66] Inhibits proliferation while promoting migration [67] Stimulates keratinocyte adhesion and wound-initiated planar migration [68]	Upregulated in Hx
Inflammatory/immunomodulatory		
IL6	Inflammatory cytokine expressed in keratinocytes [69,70] Expression of IL6 receptor suggests an autocrine regulation of keratinocyte growth [70] Stimulates proliferation in vitro [69] IL6-deficient mouse model displayed hindered cutaneous wound healing process [71]	Upregulated in Hx
TNF	TNF and its receptors are expressed by keratinocytes [72] TNF induces MMP9 production in keratinocytes [57,71,72] MMP9 regulates keratinocyte migration in a TNF-dependent manner [73]	Downregulated in Hx
TGFβ1	Known as keratinocyte growth inhibitor although it is secreted as well by keratinocytes [74,75] Found to increase expression of SERPINE1 in keratinocytes [65] Found to stimulate reepithelialization regulated by CTGF and Ras/MEK/ERK signaling pathway [76] Stimulate epithelial cell migration but not proliferation [76]	Upregulated in Hx
FGF2	Binds to FGFR2b expressed in keratinocytes [77,78] Stimulates keratinocyte migration [79] Topical application of FGF2 improved healing of traumatic tympanic membrane perforation [47]	Expressed in both at s imilar levels
FGF7	Known as an important factor in wound healing [80], which acts on keratinocytes in paracrine manner [81] Binds to FGFR2b expressed in keratinocytes [77] Stimulates keratinocyte migration [82] Inhibition of KGF receptor signaling reduces proliferation rate of keratinocytes at wound edge and delayed reepithelialization [59]	Expressed in both at similar levels
TGFα	Secreted by keratinocytes and promotes cell proliferation through autocrine signaling [83] Binds to ErbB1 receptor [78] expressed on keratinocytes [77] Induces IL6 secretion in keratinocytes indicating paracrine interaction [3]	Not expressed in ADSCs
Coagulation cascade		
F3	Regulates cell migration in a coagulation-independent manner in HaCaT [84]	Upregulated the most in Hx

those already presented in the TM repair literature. Other promoters of keratinocyte growth such as VEGFA and IL6 were upregulated by hypoxia, and secreted VEGF was increased most and so seems the most likely candidate to increase keratinocyte growth response (Table 4). The other cytokines we tested showed no significant effects of hypoxia on protein levels accumulated, so their influence remains uncertain and will require further study.

Consistent with previous findings [17,19,40,48], VEGFA protein levels in CM_{ADSC} were significantly increased by hypoxia. Of note in this study, VEGFA had been shown to be upregulated in traumatic TM perforations, in both epithelial and fibrous layers of the TM, and was reduced in nonhealing TM [49]. This could represent a novel finding for the importance of VEGFA in the healing of TM perforations and to improve healing by regulating the production of VEGFA and other factors.

The gene enrichment analysis is another approach that may provide insight; it showed a downregulation of neutrophil chemotaxis, and neutrophils are an important cell type in the inflammation phase of wound healing [50]. HIF-1α has been shown to reduce the recruitment of neutrophils in vitro through nestin-1 induction [51] and similar mechanisms may be apparent in keratinocytes. Our finding of ADSC regulated chemokines during hypoxia indicates that the paracrine effect may be directed toward regeneration through tissue repair mechanisms and reduction of inflammation.

Comparison of these data with TM wound healing microarray [52] may provide further context for these genes; however, further experiments such as proteomic analysis of the conditioned media or PCR arrays on keratinocytes posttreatment will be required to support the PCR and ELISA data. A broader transcriptome/proteome approach would also be valuable, particularly together with the rat TM wound healing transcriptome data already available [53] and our limited data set from human TM keratinocyte gene expression array. Once a set of candidates is refined, receptor inhibitors or recombinant proteins could be used to target specific pathways in vitro or in vivo.

Practical applications of this finding include cell-free conditioned media approaches by topical application in vivo or combining the mediator(s) with scaffolds to promote wound healing in a chronic TM perforation [46]. These data may also support use of fat plug myringoplasty [54,55], where direct benefits might come from ADSC present in the graft. The paracrine activity identified in this study requires further characterization and optimization regarding which tissue MSCs to use, what conditions might optimize the activity, and what is the nature of the molecular entities producing the activity. Both adipose- and bone marrow-derived MSC produce cytokines and growth factors. Adipose tissue can be a preferred source for MSC therapies, due to their low risk in isolation, high cell yields, and ready availability in most individuals.

Conclusion

In summary, this study has identified a wound healing activity from ADSCs that promotes growth and migration of tympanic membrane keratinocytes. The activity was regulated by hypoxia and was associated with expression of a number of secreted proteins that could be major effectors of keratinocyte proliferation and migration. These findings not only revealed the paracrine effects of ADSCs potentially affecting TM keratinocyte growth but also encourage further investigations for stem cells or their conditioned media as possible therapeutics for chronic TM wound healing.

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