

Type I and II Diabetic Adipose-Derived Stem Cells Respond *In Vitro* to Dehydrated Human Amnion/Chorion Membrane Allograft Treatment by Increasing Proliferation, Migration, and Altering Cytokine Secretion

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Background

- Human-derived placental tissues have been shown in randomized controlled clinical trials to be effective for healing of chronic wounds, including diabetic foot ulcers.¹⁻⁴
- Dehydrated human amnion/chorion membrane (dHACM) is a dehydrated human placental tissue allograft comprised of laminated amnion and chorion membranes.
- dHACM allografts have demonstrated the ability to modulate activity of adult stem cells *in vitro* and *in vivo*,⁵⁻⁷ where they are important for tissue maintenance and repair.⁸
- Diabetic cells may be impaired as a sequela of the disease;⁹ however, characterization of diabetic stem cells is limited.
- A specific configuration of dHACM[†] was used in this study; therefore, these results apply only to dHACM.[‡]

Purpose

The purpose of this study was to examine the ability of dHACM allografts to regulate diabetic stem cells *in vitro*.

Methods

dHACM Extracts

- Amnion and chorion layers from donated placentas were processed using a proprietary gentle cleansing process,[‡] laminated, and dehydrated under controlled conditions.
- dHACM was minced into ~1x1 mm pieces, and allowed to extract overnight at 4 °C into basal media.
- Tissue residue was removed by centrifugation, and extracts were sterile filtered.

References

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[†] EpiFix[®] amniotic membrane allograft; [‡] PURION[®] Process EpiFix[®] and PURION[®] are registered trademarks of MiMedx Group, Inc., Marietta, GA.

Methods

Proliferation

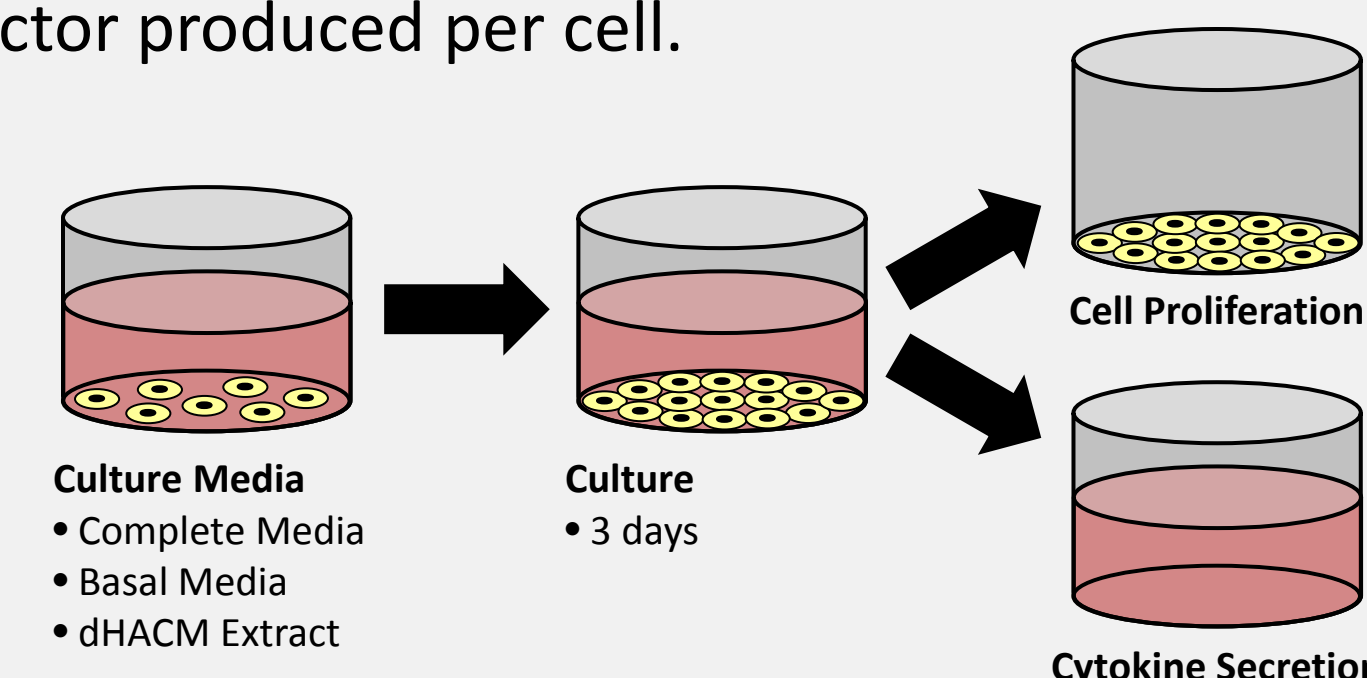
- Human adipose derived stem cells (ADSCs) from normal, type I diabetic, and type II diabetic donors were seeded at 2,500 cells/well in a 96-well plate and allowed to adhere overnight.
- Cells were rinsed with PBS, and cultured for 72 hr in complete media, basal media, or dHACM extract at 10, 5, or 1 mg/mL (Table 1).
- Cell number was determined using CyQUANT[®] Cell Proliferation Assay (Thermo Fisher), according to a standard curve of known cell quantities.

Cytokine Secretion

- Following 72 hr of culture in dHACM extract, the supernatant was recovered and tested for the presence of 80 cytokines using Quantibody[®] Multiplex ELISA Human Growth Factor and Inflammation Arrays (RayBiotech).
- Growth factor content in the starting treatments was subtracted from that in the culture media after 72 hr, and values were normalized to cell number to determine the amount of additional growth factor produced per cell.

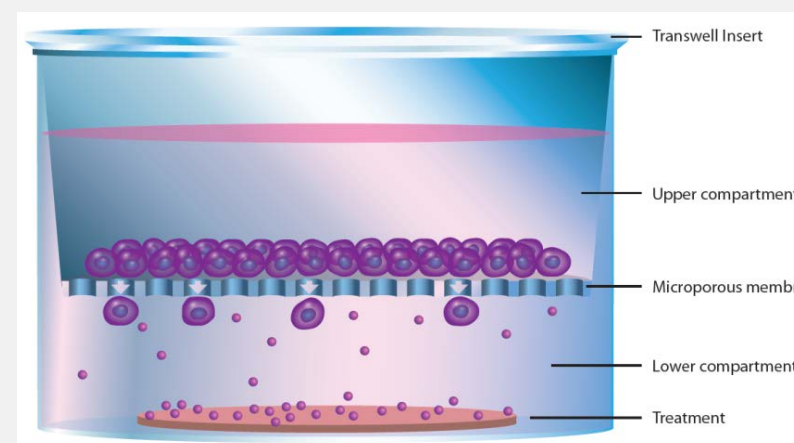
Table 1. Media formulations

	Formulation
Complete Media	Cell specific media WITH serum supplement
Basal Media	Cell specific media WITHOUT serum supplement
dHACM Extract	dHACM extracted overnight into basal media (without serum)



Migration

- Normal and diabetic ADSCs were serum-starved, treated with mitomycin C to inhibit cell division, and seeded at 40,000 cells/well in 8.0 μm pore transwell inserts.
- Treatment groups were embedded in 0.5% agarose gels in the bottom of 24-well plates at 10, 5, and 2 mg/mL in basal media, 10% newborn calf serum (positive controls), or basal media (negative control).
- After 24 hr in culture, migrated cells were either stained with DAPI or detached from the bottoms of the inserts and measured with CyQUANT assay.



Gene Expression

- Normal and diabetic ADSCs were plated at 2,500 cells/cm² in 24-well plates and treated with complete media, basal media, or dHACM extract at 10, 5, or 1 mg/mL.
- Following 5 days of treatment, RNA from ADSCs was extracted, quantified, and reverse transcribed to cDNA.
- Quantitative polymerase chain reaction (qPCR) amplification for each gene target was performed using predesigned TaqMan assays (Life Technologies), using eukaryotic 18S ribosomal RNA as an endogenous control.

dHACM Promotes Proliferation of Diabetic ADSCs

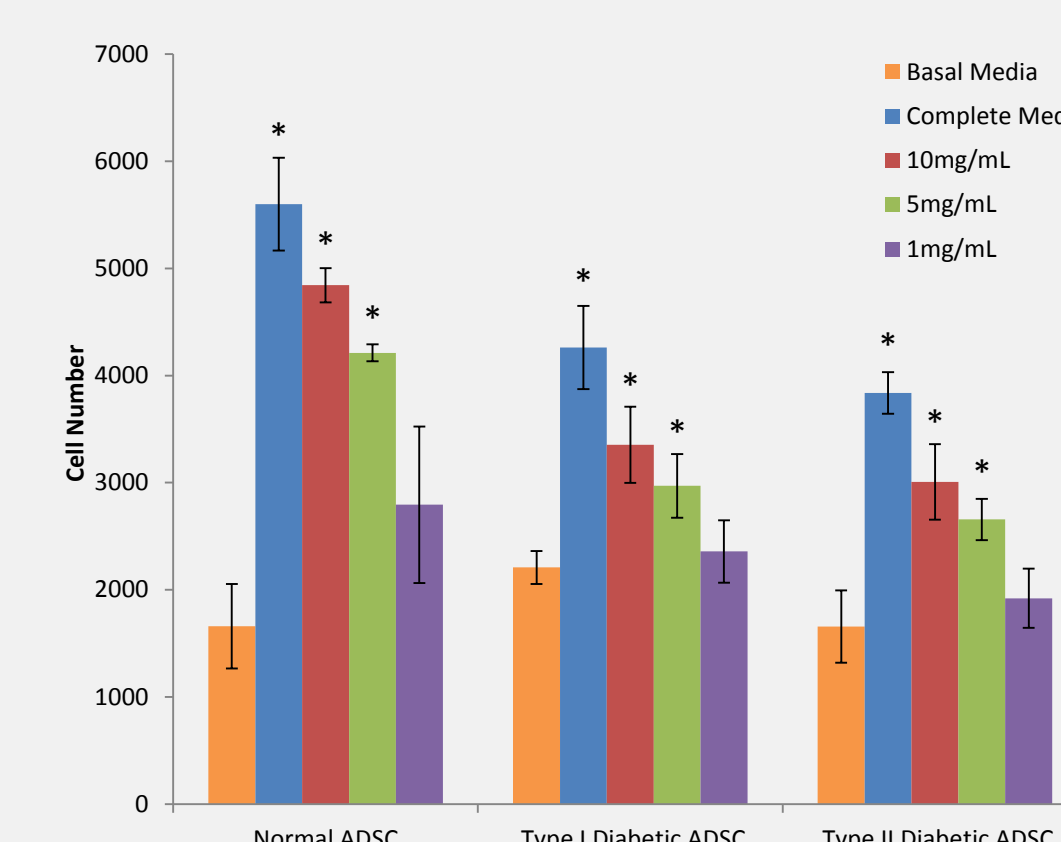


Figure 1. Proliferation of normal and diabetic ADSCs over 72 hr in response to treatment with dHACM extracts. Diabetic ADSCs exhibited an impaired proliferative response relative to normal ADSCs; however, incubation with dHACM extracts resulted in **increased proliferation of normal and diabetic ADSCs in a dose-dependent manner**. * indicates $p < 0.05$ compared to basal controls (n=5).

dHACM Modulates Cytokine Secretion by Diabetic ADSCs



Figure 3. Cytokine secretion by normal and diabetic ADSCs in response to treatment with dHACM extracts. The data are presented as a heat map with upregulation relative to basal media represented as a green color gradient and downregulation represented as a red color gradient. Normal, type I diabetic, and type II diabetic ADSCs **modulated secretion of various soluble signals** over basal controls in response to dHACM treatment, including **regulators of inflammation, angiogenesis, and healing**.

Results

dHACM Stimulates Migration of Diabetic ADSCs

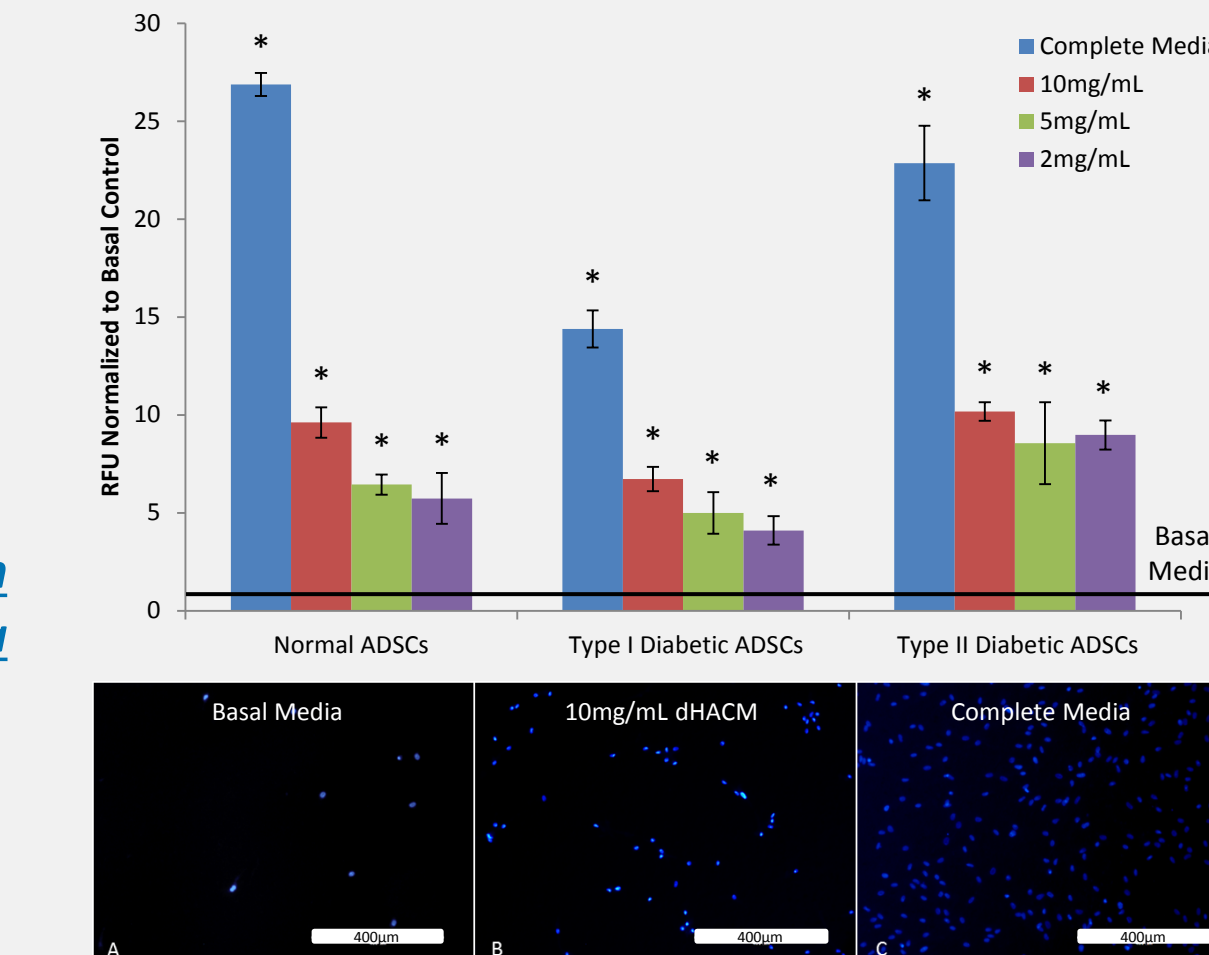


Figure 2. Migration of normal and diabetic ADSCs over 24 hr in response to treatment with dHACM extracts. Type I diabetic cells experienced lower migration compared to normal ADSCs in complete media; however, normal and diabetic ADSCs were stimulated by dHACM to **increase migration in a dose-dependent manner**, relative to basal controls. Relative fluorescent units (RFUs) represents the number of migrated cells, normalized to basal controls (black line = 1). DAPI staining of migrated cells visually confirmed the migration response. Representative images of normal ADSCs in response to (A) basal medium, (B) 10 mg/mL dHACM extract, and (C) serum are shown. * indicates $p < 0.05$ compared to basal controls (n=5).

dHACM Regulates Gene Expression of Diabetic ADSCs

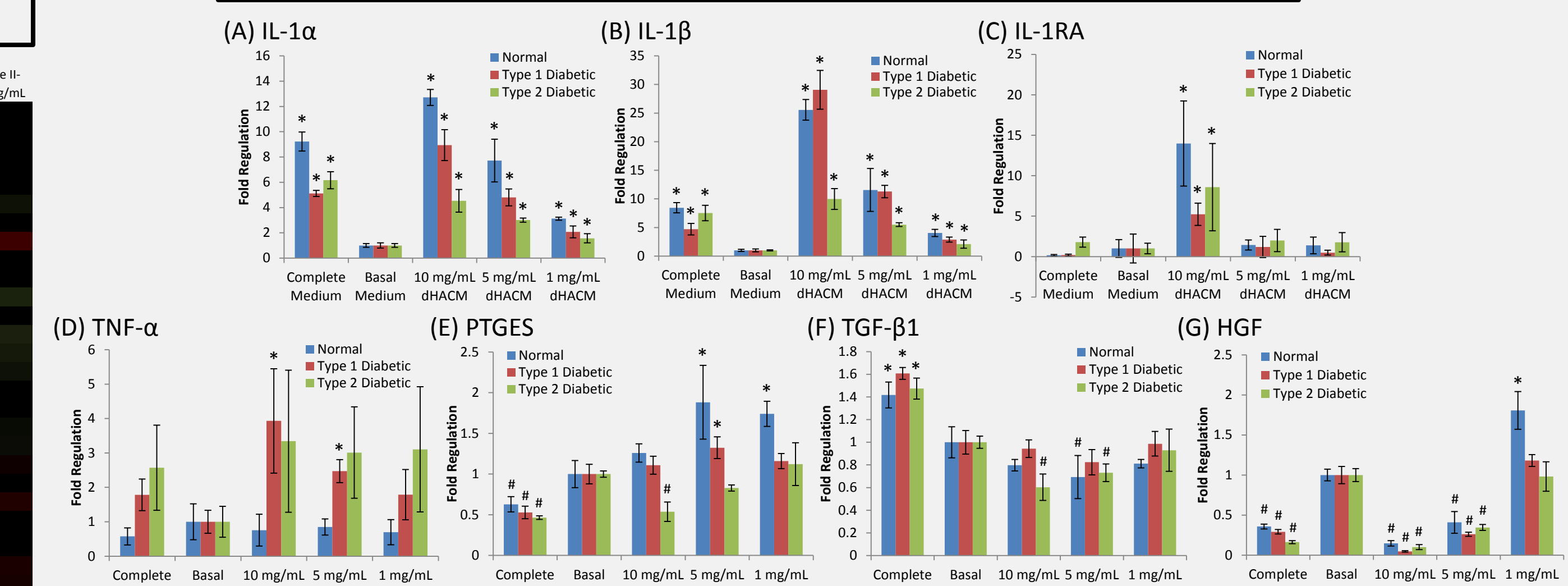


Figure 4. Gene expression of normal and diabetic ADSCs in response to treatment with dHACM extracts. Diabetic ADSCs demonstrated upregulation of gene targets related to immunomodulation, relative to normal ADSCs. Normal and diabetic ADSCs responded to dHACM treatment after 5 days by **regulating expression of genes related to immunomodulation**. Fold regulation of (A) interleukin 1α (IL-1α), (B) IL-1β, (C) IL-1 receptor antagonist (IL-1RA), (D) tumor necrosis factor-α (TNF-α), (E) prostaglandin E synthase (PTGES), (F) transforming growth factor β1 (TGF-β1), and (G) hepatocyte growth factor (HGF) is expressed relative to ADSCs cultured in basal medium (fold regulation=1). **Expression of IL-1α, IL-1β, as well as IL-1 receptor antagonist (IL-1RA) was commonly upregulated** by all ADSCs following dHACM treatment in a dose-dependent manner. * indicates upregulation and # indicates downregulation compared to basal controls ($p < 0.05$; n=5).

Conclusions

dHACM stimulated diabetic ADSCs to migrate, proliferate, and alter cytokine expression including immunomodulatory cytokines, suggesting that, despite their diabetic origin, ADSCs may respond to dHACM to enhance diabetic wound healing. Both traditional pro-inflammatory and anti-inflammatory factors were upregulated following dHACM treatment, suggesting that dHACM may modulate inflammation in diabetic wounds by stimulating ADSCs to regulate the critical balance of immunomodulatory signals necessary to promote healing.