Title

Gene Expression Studies Pertaining to Extracellular Matrix Integrity and Remodeling: Nuances and Pitfalls of In Vitro Investigations

Journal

Journal of Drugs in Dermatology, 18(12)

ISSN

1545-9616

Authors

Widgerow, Alan D
Garruto, John A

Publication Date

2019-12-01

Peer reviewed

Gene Expression Studies Pertaining to Extracellular Matrix Integrity and Remodeling: Nuances and Pitfalls of In Vitro Investigations

Alan D. Widgerow MBBCh MMed (Plast) FCS FACS,[a] John A. Garruto BS[b]

[a]Center for Tissue Engineering, Department of Plastic Surgery, University of California, Irvine, CA; Alastin Skincare Inc., Carlsbad CA

[b]Free Radical Technology, Inc, Oceanside, CA

ABSTRACT

Anti-aging strategies using topicals with active agents demand validation and proof of efficacy. One investigation in this realm involves gene expression testing. This study undertakes gene expression analysis of Alastin Skincare Regenerating Skin Nectar (RSN) using an in vitro human skin model. The current study is similar to other published human skin model studies, but with additional time periods beyond 24-hours (which are more appropriate for testing peptides) and a suitable control for the Alastin non-aqueous product. Results show the Alastin product upregulates a large array of genes within areas of skin renewal, extracellular matrix remodeling, barrier function, and inflammation after 72 hours. The study provides gene expression data that support the clinical success of the product. It also demonstrates the difficulty and vulnerabilities in assessing efficacies of products with certain in vitro investigations when the nuances of that product are not considered.

J Drugs Dermatol. 2019;18(12):1255-1259.

INTRODUCTION

Skin renewal and regeneration are paramount to efforts in anti-aging strategies and rejuvenation. This is particularly relevant in a milieu of extracellular matrix (ECM) distortion and disruption brought about by extrinsic (photodamage) and intrinsic (aging) factors that initiate and maintain these changes.[1,2] In an effort to counteract changes within the ECM and epidermis, peptides, growth factors, and other active ingredients have been used. In particular, a new generation of peptides has provided solutions to many challenges presented by biologic materials such as growth factors. Peptides' very low molecular weights ensure absorption, synthetic manufacturing processes generate a predictable quality and quantity of stable actives, far lower amounts of potentially cytotoxic preservatives are needed for shelf life maintenance, and peptides have an increased safety profile related to unexpected cellular proliferation.[3]

When comparing efficacies of growth factors versus peptides, certain functional nuances need to be borne in mind when testing gene responses, particularly in laboratory environments. In that context, a recent study[4] attempted to compare various formulations including growth factor- and non-growth factor-based skin care products, assessing their biologic activity based on gene expression responses. Major limitations were identified related to this study, which included the following:

- A single test period was examined (24 hours), which, while this time frame may be suited to growth factor stimulation, peptides behave differently and have been demonstrated in many cases to have maximal activity in gene expression at 48, 72, and even longer time periods.

- When selecting controls, water may be ideal for certain aqueous formulations but for non-aqueous anhydrous preparations such as (Alastin Regenerating Skin Nectar), this provides potential inaccuracies.

- Finally, although the skin culture EFT model selected has been successfully used as a surrogate for human studies, there are inherent limitations to testing combined cell lines. Although transcription (cell commands) signaling may be demonstrated, the true proof of efficacy often resides in the translational capacity of the product, that relating to new cell and protein regeneration. This is best represented by histological assessments of biopsies which take into account the ability of the actives to penetrate skin in a real world environment.

In an effort to address some of the limitations addressed above, a study was designed to test Alastin Skincare Regenerating Skin Nectar, ensuring analysis at two time points, selecting appropriate baseline control comparators, and using a broad panel of genes (Genemarkers Standard Skin Panel) covering a range of important biological functions in skin rather than a few selected genes.

MATERIALS AND METHODS

As in the previous study,[4] EpiDermFT™ 3D full-thickness in vitro skin culture models containing epidermal and dermal cell layers (EFT-400, MatTek Corp.) were used. Gene expression was assessed in full-thickness tissues following a 24- or 72-hour exposure to the test material. The following treatment groups were included (N=4):

- Alastin Skincare Regenerating Skin Nectar: (Alastin Product)
- Alastin Skincare Regenerating Skin Nectar Base (non-aqueous control): (Alastin Base)

EFT-400 tissues were stored at 4°C. The day before treatment, tissues were equilibrated overnight at 37°C with 5% CO2 and ~95% relative humidity using a 2.5mL volume of EFT-400 culture medium. The following day, equilibration medium was removed from each EFT-400 culture well and replaced with 5.0mL fresh EFT-400 medium (the vendor-recommended volume for a 72-hour study duration). Tissues and culture media were collected after 24, 48, or 72 hours. Test medium from each well was collected and stored at -80°C until the lactate dehydrogenase (LDH) assay was performed. Each EFT-400 culture was cut into quarters and placed into a tube containing RNAlater preservative solution for a 2-hour incubation at room temperature. Tissues in RNA later were then stored at 4°C for a 2-4-day incubation prior to RNA isolation. The following sequence was adopted:

1. *LDH Cytotoxicity Assay:* Standard assay for cytotoxicity was performed.
2. *RNA Isolation:* RNA was isolated from each tissue using a Maxwell 16 Simply RNA Tissue kit (Promega) following the manufacturer's instructions.
3. *cDNA Synthesis:* cDNA was generated using a High Capacity cDNA Synthesis Kit according to the manufacturer's instructions (Applied Biosystems). Standard Skin Panel gene cDNA was generated from 2000 ng RNA per sample.
4. *qPCR Processing:* qPCR reactions were run using validated Taqman® gene expression assays. Open Arrays were run in a Life Technologies QuantStudio 12K Flex instrument. Each gene was assayed in duplicate.

Statistical Data Analysis: Statistical analysis was performed using the relative quantitation (RQ) method. In the first step of an RQ analysis, the CT value of the target gene is normalized to the CT value of an endogenous control gene to generate the delta CT (dCT). dCT values are calculated in order to normalize for variability between the samples that may occur during the experimental procedures. Statistically significant (unpaired t-test, $P<0.05$, N=4) changes in gene expression are shown as RQ values.

RESULTS

Cytotoxicity Data

LDH is a cytoplasmic enzyme released into the culture medium of damaged or dying cells. The Alastin Product and Alastin Base both showed <20% cytotoxicity at all 3 time points demonstrating good profiles for cellular viability (Figure 1).

Gene Expression Data

For the Alastin Product vs Alastin Base, statistically significant changes in gene expression occurred with the most beneficial response at 72 hours. The Alastin Product produced beneficial changes in gene expression related to antioxidant protection, cell renewal and regeneration, extracellular matrix integrity and anti-inflammatory response. It is noteworthy that results demonstrate poor responses of gene expression at 24 hours confirming previous results of peptide studies that showed efficacy at 72 hours and emphasizing the importance of presenting gene expression analysis in an appropriate study design related to the nuances of the active agents being tested (Figures 2 A, B).

FIGURE 1. Relative % cytotoxicity [LDH Activity Assay].

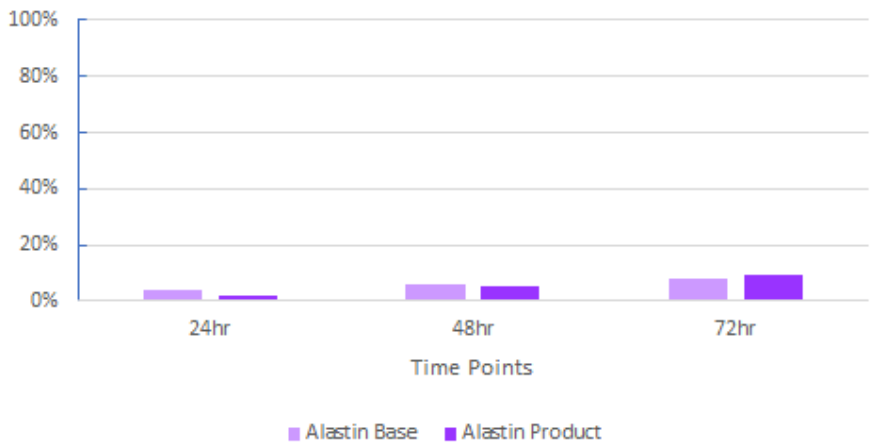
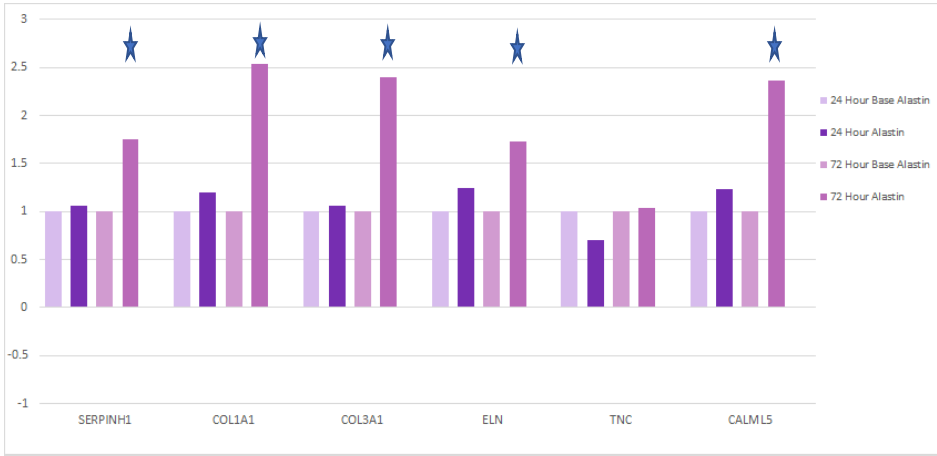
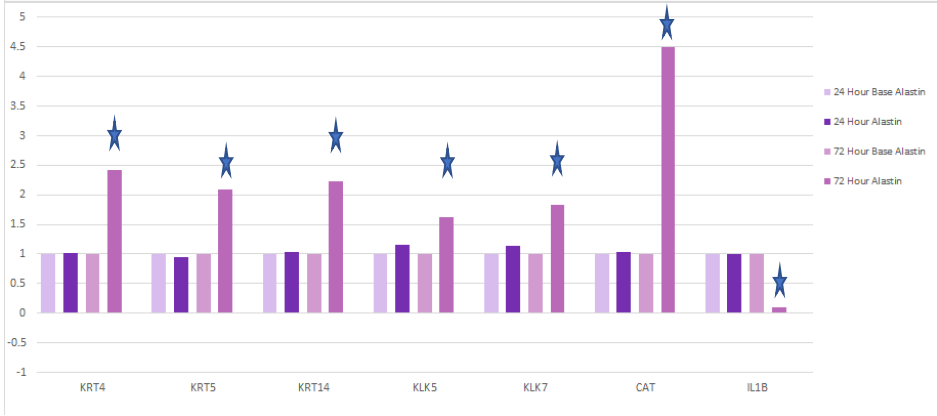


FIGURE 2A AND 2B. Statistically significant changes in gene expression induced by Alastin RSN at 72 hours.

(2A)



(2B)



Statistics

Unpaired t-tests were carried out using TIBCO Spotfire software. Statistically significant results ($P<0.05$) were reported. The statistical comparison generated delta delta CT [ddCT] values (the mean dCT of the treated group – the mean dCT of the control group). The statistical software converts the ddCT values into log and linear RQ values for export [RQ = 2-ddCT]. The linear RQ values were then converted to linear fold-change values to simplify data interpretation.

DISCUSSION

The science related to anti-aging formulations has undergone major advances in recent years. Peptides are considered reliable and effective transformers of skin homeostasis through signaling mediations, cellular proliferation, and regenerative capacities. Validation of these effects is an important part of the proof of concept through a series of steps, often beginning with gene expression analysis. However, as demonstrated in this study, certain nuances characteristic of peptide behavior require consideration when validating efficacy of these molecules. By extending the period of testing and selecting appropriate controls, significant changes in gene expression have been demonstrated.

Thus, the TriHex Technology and overall formulation of Alastin Regenerating Skin Nectar has been demonstrated to significantly up-regulate gene expression in the critical areas of cell renewal, ECM remodeling and barrier function at 72 hours. These results contrasted with a previous study that tested the product with water as the control and only at the 24-hour time point.

In particular, the array of genes tested (Table 1) involve skin function from structural support and integrity of the extracellular matrix in the dermis to the cell renewal function and barrier function of the more superficial epidermis thus providing comprehensive assessment of the entire skin layer (Table 1). The range of genes tested reflect the functional homeostasis at various levels of the skin structure. Thus, protection at the surface of the skin relates to barrier function, covering various aspects from thermoregulatory issues, transepidermal water loss, microbial invasion, etc – genes measured here – calmodulins and kallikreins. Moving from surface protection to cell renewal and replacement particularly at a superficial level, relevant genes are keratins and tenascin. Then the ECM structural components are particularly important in ensuring the foundation of skin

TABLE 1.

Actions of Genes Assessed in Standard Skin Panel		
GENE	**Detailed Function**	**Biological Function Skin**
SERPINH1	Plays a role in collagen biosynthesis as a collagen-specific molecular chaperone. It codes for heat shock protein HSP47 indispensable for the proper folding of collagen triple helix.[11]	Extracellular Matrix Integrity
COL1A1	Col1A1 encodes the pro-alpha1 chains of type I collagen whose triple helix comprises two alpha1 chains and one alpha2 chain. Type I is a fibril-forming collagen found in most connective tissues and is abundant in the dermis where it is the most critical structural protein.[12]	Extracellular Matrix Integrity
COL3A1	COL3A1 gene provides instructions for making type III collagen abundant in the skin particularly in the early phases of wound healing. Ratio conversion to increased type 1 versus type 3 collagen is sought after for controlled scar formation.[12,13]	Extracellular Matrix Integrity
ELN	This elastin gene encodes protein components of elastic fibers in the extracellular matrix important for conferring elasticity tissues including the skin.[12]	Extracellular Matrix Integrity
TNC	Tenascin genes modulate cell adhesion and cellular responses to growth factors and is down regulated with skin aging.[14,15]	Cell Renewal/ Regeneration
CALML5	Encodes for calmodulin protein and regulates epidermal differentiation and barrier function.[16]	Barrier Function
KRT4 KRT5 KRT14	Keratins are intermediate filament proteins that have essential functions in maintaining the structural integrity of epidermis and improves barrier function.[17]	Cell Renewal/ Regeneration/Barrier function
KLK5 KLK7	Kallikrein peptidases are responsible for coordinating desquamation and renewal of epithelial layers and regulating stratum corneum and barrier function.[18]	Extracellular Matrix Breakdown/ Barrier function
CAT	Catalase is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS)very representative of anti-oxidant activity.[19]	Antioxidant
IL1-β	This interleukin cytokine is one of the most important mediators of the inflammatory response needing constant control.[20]	Inflammation

homeostasis is maintained and cross talk between fibroblasts and structural proteins is ensured – here appropriate genes measured are the collagens, elastin, and tenascin. Finally, as a general homeostatic gauge of antioxidant and anti-inflammatory activity, catalase and Interleukin 1 beta are representative of these functions (catalase upregulation important for mopping up reactive oxygen species and IL-1b down regulation ensuring balanced, controlled inflammation). This general choice of genes was felt to be representative of overall skin function and homeostasis.

The primary goal of this study was to demonstrate efficacy of the product with an appropriate test model design. These results confirm the efficacy of Alastin Nectar with regards to gene expression. In addition, as has been previously noted, gene expression is the first of many validation steps, and it may be argued that histological confirmation and clinical studies are more important in assessing efficacy. This has been carried out and reported in multiple publications.[2,5-10]

Aside from the favorable gene expression demonstrated here by the Alastin product, the issue of preservative interplay in cellular well-being is an area that may deserve more attention. It is known that biologic products such as growth factors need considerably more preservatives added to preparations than active peptides, in order to ensure shelf stability. Alastin Nectar contains no preservatives, which makes it especially suited for application to ablated and post-procedure skin. This is an advantage compared to growth factor preparations, which have the potential concerns of large molecular weight, inconsistent batch quality, unpredictable biologic effects, and an unfocused targeted approach that accompany biologic/growth factor technologies.

CONCLUSION

Gene expression studies are a necessary first step in determining the potential for clinical efficacy of products used in anti-aging skin care. Parameters chosen for testing these formulations need to match the physical nature and behavior characteristics of the active agents involved. To this end, this study was performed in response to a previous report in JDD[4] and was designed to address the inherent characteristics of a peptide-based, anhydrous Alastin Skin Nectar formulation. The same EFT model was used as in the prior study, however, additional testing time points (72 hours) and a more appropriate non-aqueous control were added to the protocol. Results convincingly demonstrated significant upregulation in gene expression of Alastin Nectar in a wide array of genes in areas of cell renewal, regeneration, ECM remodeling, and barrier function. Combined with multiple other published studies demonstrating histological changes and consistent clinical results, this study adds to the validity of Alastin Skin Nectar as a potent pre-conditioner, wound healing and anti-aging agent, reaffirming its ongoing success in clinical cases.

DISCLOSURES

Dr Widgerow is Chief Medical Officer of Alastin Skincare. Mr Garruto is a paid consultant of Alastin Skincare. Genemarkers company is an independently operated company unaffiliated with Alastin Skincare.

ACKNOWLEDGMENT

Genemarkers (Kalamazoo, MI) for in vitro testing and Firma Clinical Research, (Chicago, IL) for statistical analysis.

REFERENCES

1. Flament F, Bazin R, Laquieze S, Rubert V, Simonpietri E, Piot B. Effect of the sun on visible clinical signs of aging in Caucasian skin. *Clin Cosmet Investig* Dermatol. 2013;6:221-232.
2. Widgerow AD, Fabi SG, Palestine RF, et al. Extracellular matrix modulation: optimizing skin care and rejuvenation procedures. *J Drugs Dermatol.* 2016;15(4s):S63-S71.
3. Gorouhi F, Maibach HI. Role of topical peptides in preventing or treating aged skin. *Int J Cosmet Sci.* Oct 2009;31(5):327-345.
4. Kadoya K, Makino ET, Jiang LI, et al. Upregulation of extracellular matrix genes corroborates clinical efcacy of human fibroblast-derived growth factors in skin rejuvenation. *J Drugs Dermatol.* 2017;16(12):1190-1196.
5. Robinson D, Frulla A. Randomized, split-face/décolleté comparative trial of procedure enhancement system for fractional non-ablative laser resurfacing treatment. *J Drugs Dermatol.* 2017;16(7):707-710.
6. Vanaman Wilson MJ, Bolton J, Fabi SG. A randomized, single-blinded trial of a tripeptide/hexapeptide healing regimen following laser resurfacing of the face. *J Cosmet Dermatol.* Mar 31 2017.
7. Widgerow AD, Jiang LI, Calame A. A single-center clinical trial to evaluate the efficacy of a tripeptide/hexapeptide antiaging regimen. *J Cosmet Dermatol.* Mar 4 2018.
8. Nelson A, A O. A tripeptide/hexapeptide anti-aging regimen that targets both collagen and elastin, and improves both physician and subject scoring of facial aesthetics. *Case Rep Dermatol.* 2018;3(1):1-5.
9. Reivitis A, Karimi K, Griffiths C, Banayan A. A single-center, pilot study evaluating a novel TriHex peptide- and botanical-containing eye treatment compared to baseline. *J Cosmet Dermatol.* Jun 2018;17(3):467-470.
10. Calame A, AD W. Histological changes associated with extracellular matrix-remodeling topical therapy. *Case Rep Dermatol.* 2017;2(2):1000126.
11. Ono T, Miyazaki T, Ishida Y, Uehata M, Nagata K. Directin vitroandin vivoevidence for interaction between Hsp47 protein and collagen triple helix. *J Biol Chem.* 2012;287(9):6810-6818.
12. Oikarinen A. Connective tissue and aging. *Int J Cosmet Sci.* 2004;26(2):107-107.
13. Widgerow AD. Cellular/extracellular matrix cross-talk in scar evolution and control. W*ound Repair Regen.* 2011;19(2):117-133.
14. Chiovaro F, Chiquet-Ehrismann R, Chiquet M. Transcriptional regulation of tenascin genes. *Cell Adh Migr.* 2015;9(1-2):34-47.
15. Park JS, Lee YH, Lee DH, Chung JH, Lee S-T. Expression of tenascin-C is down-regulated during intrinsic skin aging. *J Dermatol Sci.* 2017/05/01/ 2017;86(2):e92-e93.
16. Sun B, Boxer L, Ransohoff J, et al. CALML5 is a ZNF750- and TINCR-induced protein that binds stratifin to regulate epidermal differentiation. *Genes Dev.* 2015;29:2225–2230.
17. Torma H. Regulation of keratin expression by retinoids. *Dermatoendocrinol.* Jul 2011;3(3):136-140.
18. Lundstrom A, Egelrud T. Stratum corneum chymotryptic enzyme: a proteinase which may be generally present in the stratum corneum and with a possible involvement in desquamation. (0001-5555 (Print)).
19. Chelikani P, Fita I, Loewen PC. Diversity of structures and properties among catalases. *Cell Mol Life Sci.* 2004;61(2):192-208.
20. Afonina IS, Muller C, Martin SJ, Beyaert R. Proteolytic Processing of interleukin-1 family cytokines: variations on a common theme. *Immunity.* 2015;42(6):991-1004.

AUTHOR CORRESPONDENCE

Alan D. Widgerow
E-mail:..... awidgero@uci.edu