ML-05 (Detoxified Streptolysin O): Effects on Extracellular Matrix and Immune Response Gene Expression in Pathway-focused DNA Microarrays

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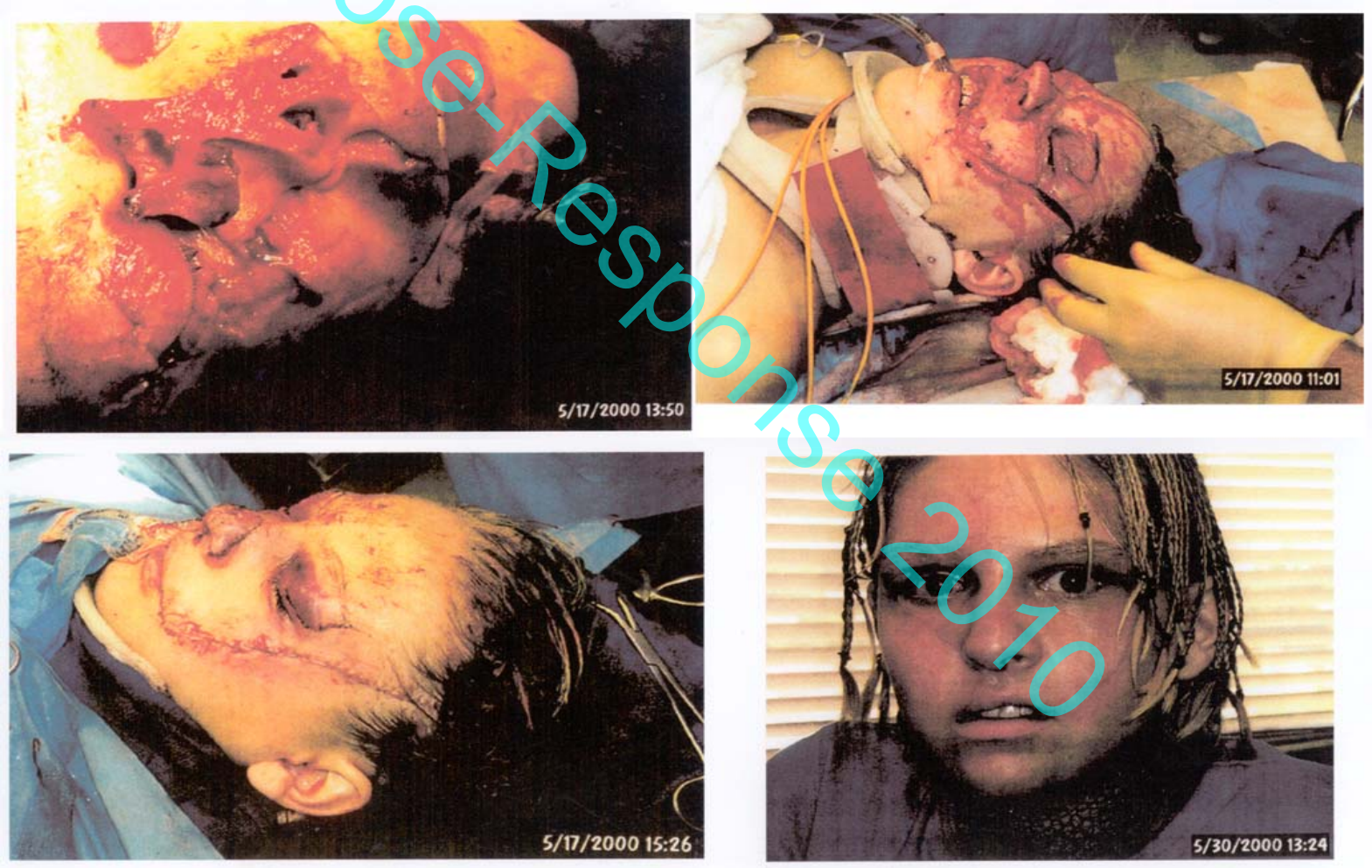
Streptolysin O (SLO) and ML-05

- SLO - ~65 kDa protein hemolytic exotoxin produced by *Streptococcus* species of groups A, C, and G

- Acts as a thiol-activated, cholesterol-binding agent that can form pores in cell membranes in its reduced state

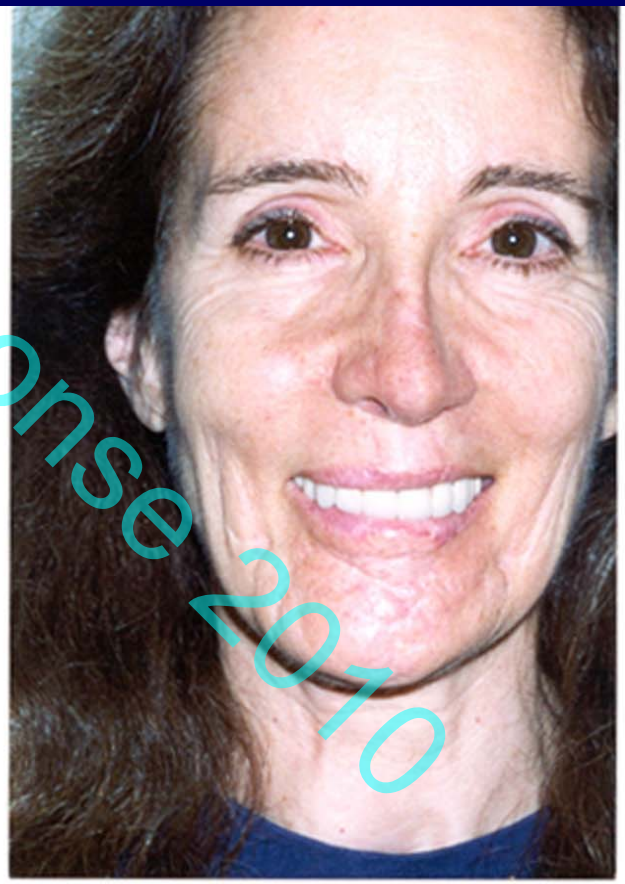
- ML-05 = Detoxified SLO
  - Modulation of collagen levels in murine models of fibrosis (Mamber et al., Nonlinearity Biol Toxicol Med. 2:67–87, 2004)
  - Increased expression of hyaluronan receptor CD44 (Mamber et al.)
  - Increased keratinocyte migration/proliferation *in vitro* and enhanced wound healing *ex vivo* (Tomic-Canic et al., Wound Repair Regen. 15:71-79, 2007)

ML-05 Administered on Compassionate Use Basis After Car Accident

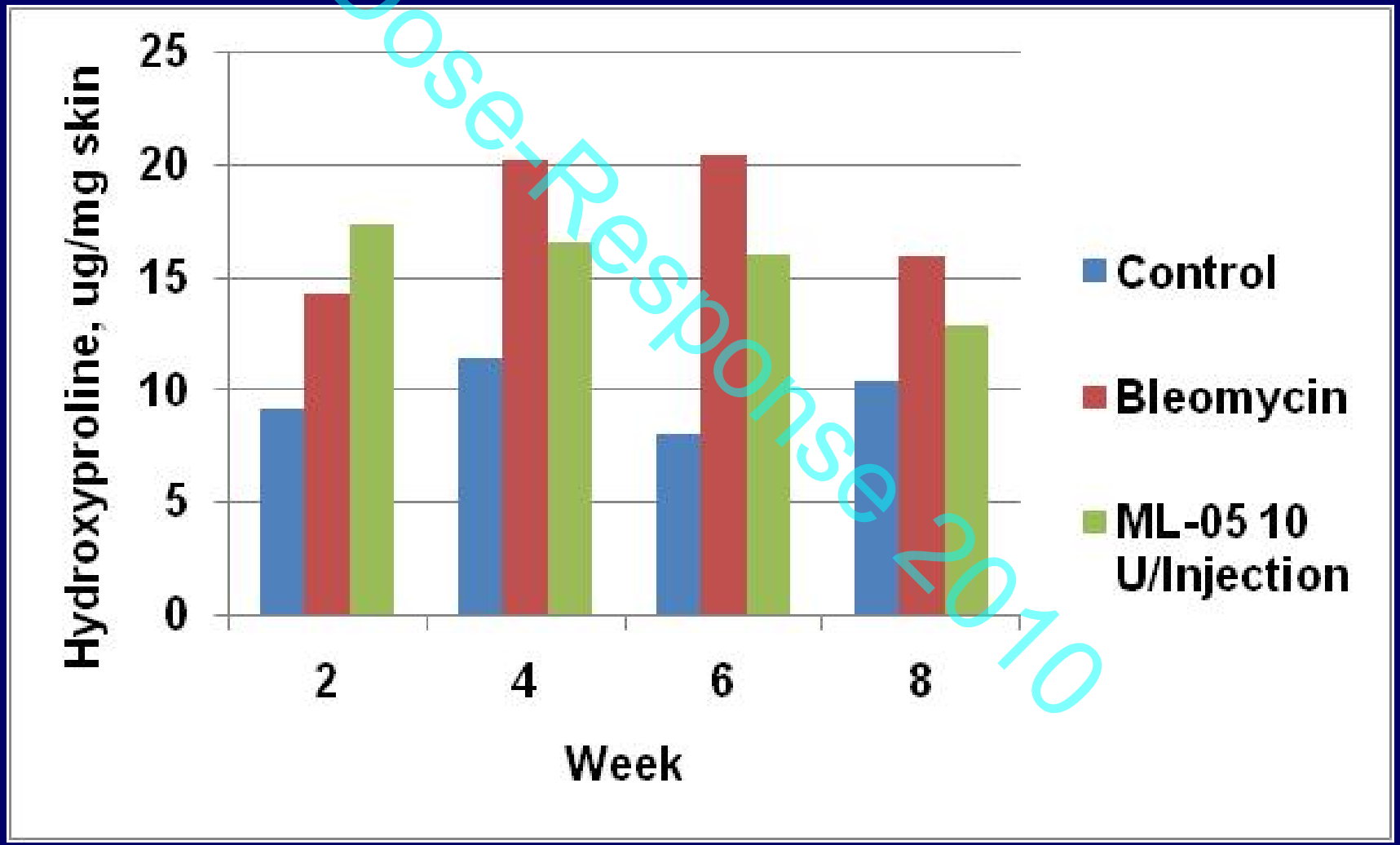


Patient About 2 Months Later

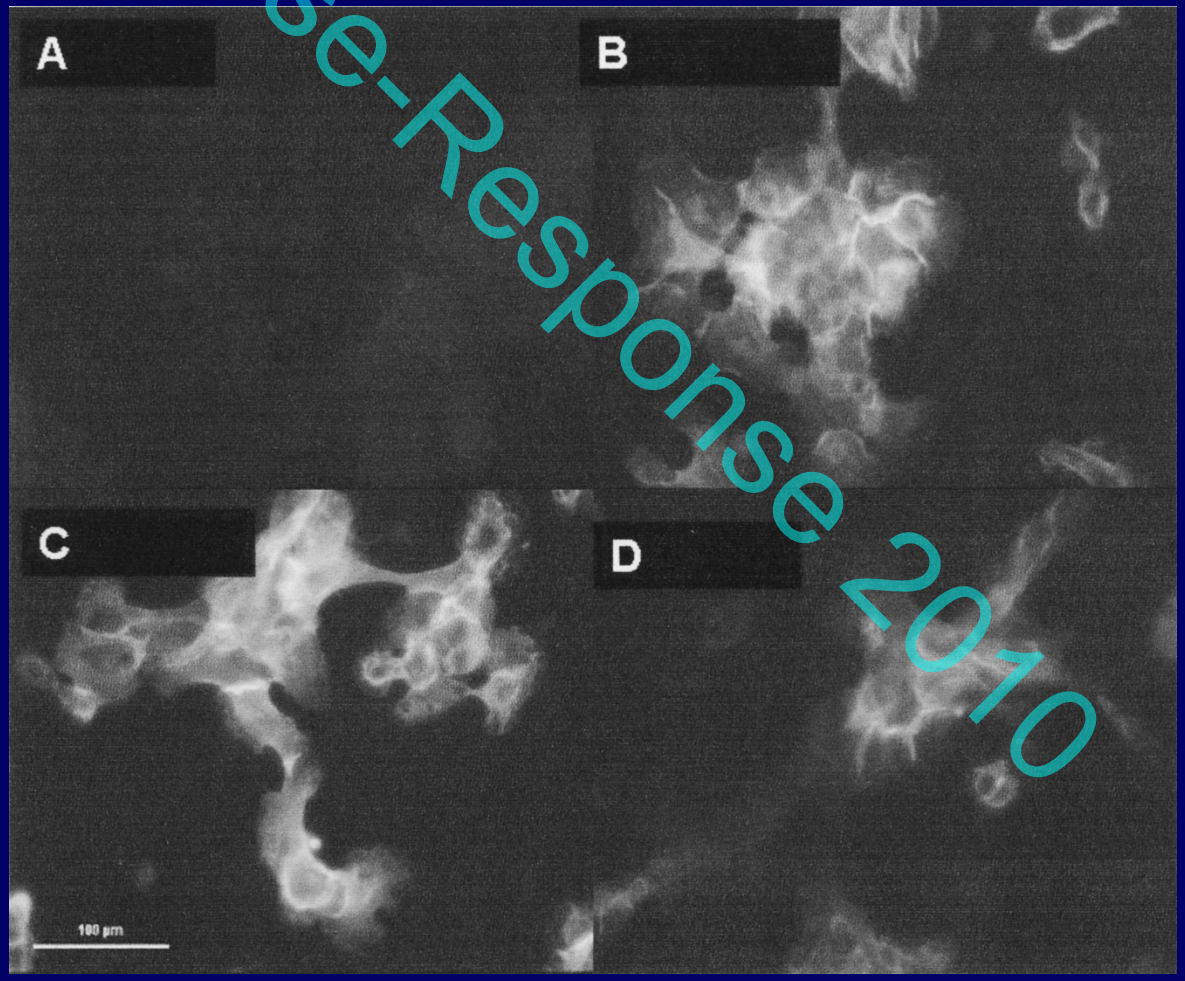
Scar Reduction Years After Accident/Skin Graft Surgery



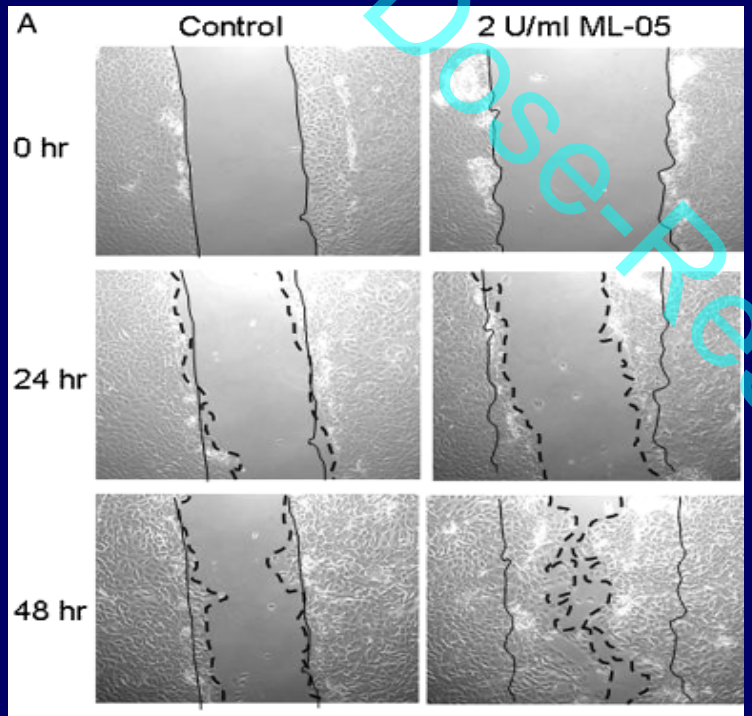
Hydroxyproline Assay Results from Bleomycin-Induced Fibrosis Study



Effects of ML-05 (0.02–2 units/ml) on Cell Surface Marker CD44of Keratinocytes after 26 hr Exposure (immunofluorescence staining of CD44)



Effects of ML-05 on Keratinocyte Migration and Proliferation (Tomic-Canic et al., 2007)

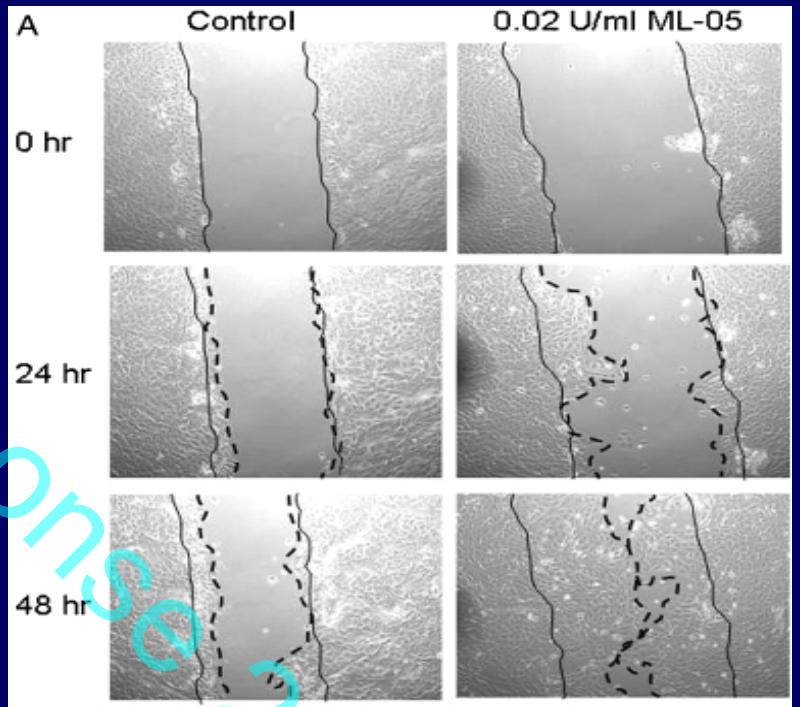


B

% Wound width coverage

ML-05 U/ml	24 hrs	48 hrs
0	5.0± 1	16.0± 2
0.02	8.7± 1 *	32.6± 1*
0.2	6.1 ± 3	58.0± 4*
2	36.7 ± 3*	81.6± 2*
20	21.3 ± 2*	40.4± 1*

+ mitomycin C



B

% Wound width coverage

ML-05 U/ml	24 hrs	48 hrs
0	15.0± 1	40.0± 2
0.02	23.9± 2 *	87.0± 3*
0.2	29.0± 2 *	75.0± 4*
2	8.3± 1 *	38.9± 3
20	12.2± 1	20.4± 1*

- mitomycin C

Research Objective

The literature suggests that ML-05 may promote keratinocyte migration/proliferation, enhance wound healing and modulate collagen levels through extracellular matrix (ECM)-related physiological, immunological and enzymatic (collagenolytic) mechanisms. It was thought that gene expression profiling technologies could be employed to identify differentially expressed genes involved in mechanisms of action of ML-05. ***Accordingly, the objective of this research was to evaluate the effects of ML-05 on ECM and immune response gene expression in primary human keratinocytes using pathway-focused DNA microarrays and related technologies.***

Pathway-focused DNA Microarrays

Pathway-focused DNA microarrays, such as the Oligo GEArrays® available from SABiosciences of Frederick MD, represented a useful, cost-effective alternative to complete genome arrays. Each microarray contains a panel of genes involved in a particular biological pathway or disease state. The procedures used with these microarrays yield data reflecting levels of the expression of the genes of interest.

Microarrays of Interest

1) Extracellular Matrix (ECM) microarrays – contain over 100 target DNA sequences for genes known to be associated with the biological structure and function of the extracellular matrix.
2) Autoimmune and Inflammatory Response microarrays – contain over 400 DNA sequences for common (cell-mediated and humoral) cytokine production and metabolism genes, plus those for inflammatory cytokines, chemokines, receptors, and proteins involved in cytokine-cytokine receptor interactions.

How Pathway-focused Microarrays Work

Source Material - RNA from cells, tissues, etc.

Experiment – Treatment and control groups, followed by total RNA extraction and purification.

RNA preparation - total RNA enzymatically converted to labeled cDNA (complementary DNA) or cRNA.

Microarrays - a set of distinct, gene-specific, nucleic acid probes immobilized on a solid support.

Hybridization - of labeled cRNA to the immobilized nucleic acid probe.

Detection - each gene-specific spot is detected using chemiluminescent methods (CCD camera). The signal produced at each spot is representative of the amount of message in the original RNA sample.

Cell Treatment Procedures

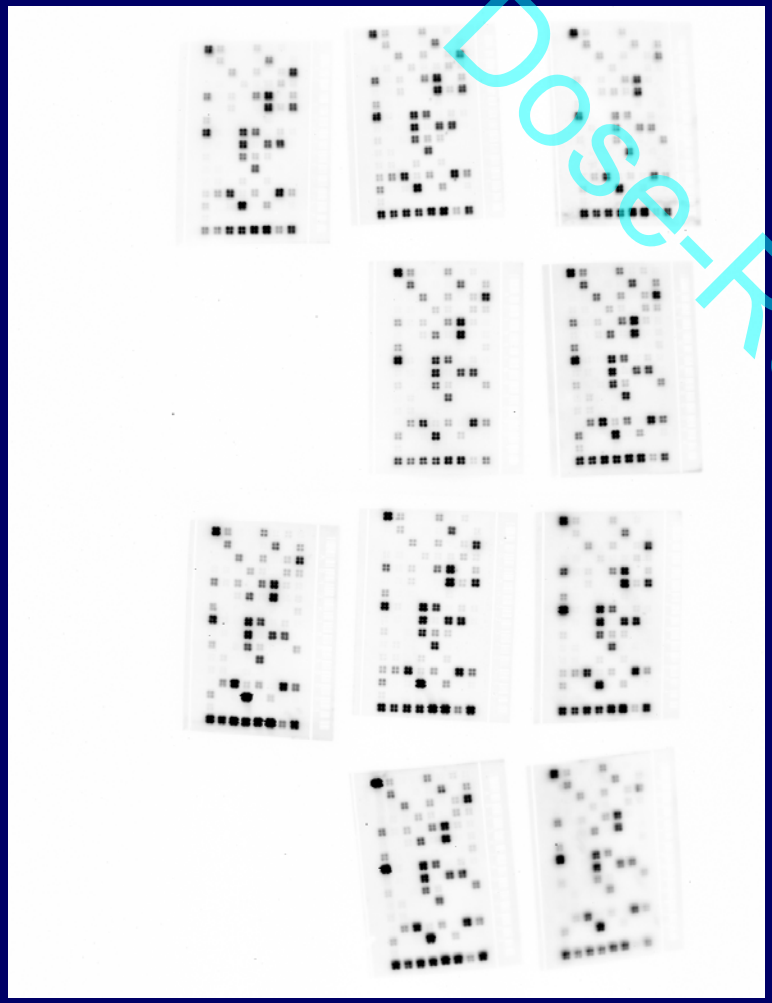
1) All primary cells: NHEK (normal human epidermal keratinocytes), NHDF (normal human dermal fibroblasts) and PBMC (peripheral blood mononuclear cells) – 2nd- or 3rd-passage cells grown in T-25 flasks using media from supplier of cells.

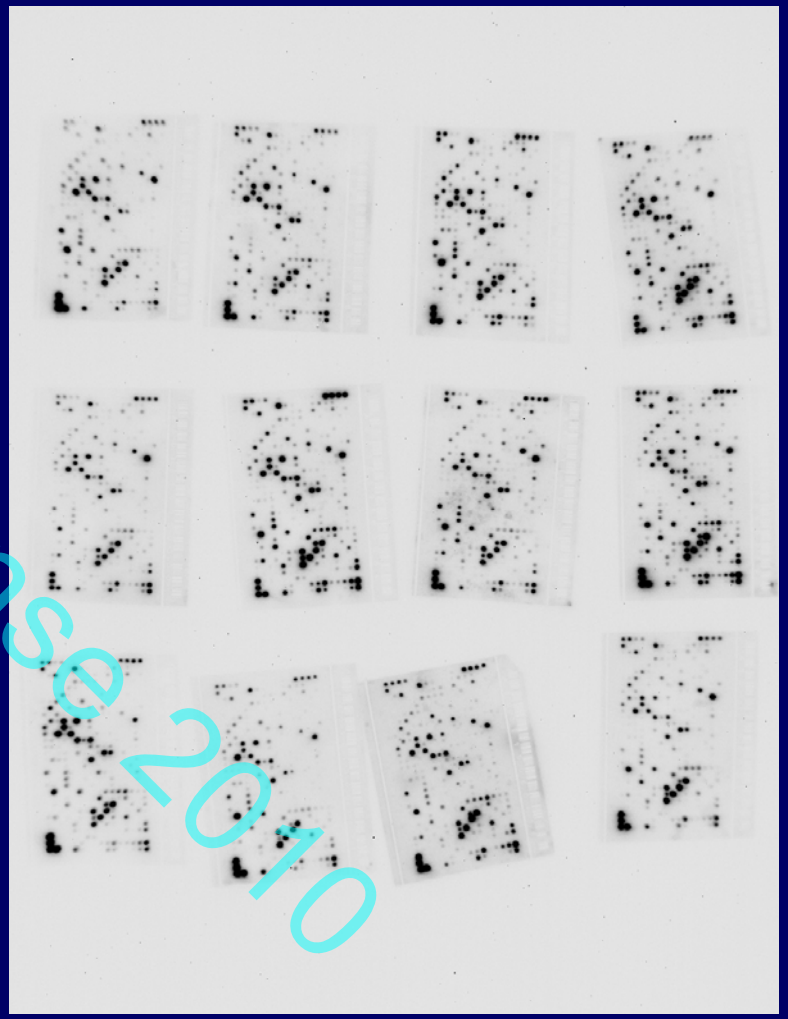
2) Cells treated with 9 concentrations of ML-05 for 24 hours prior to RNA isolation, plus vehicle control; 0.000002, 0.00002, 0.0002, 0.002, 0.02, 0.2, 2, 20, 200 IU/ml.

3) Pattern-specific microarrays of interest: a) Extracellular Matrix (about 100 genes) and b) Autoimmune and Inflammatory (over 400 genes).

4) Three replicates per data point for statistical and publication purposes .

Typical Sets of Microarrays





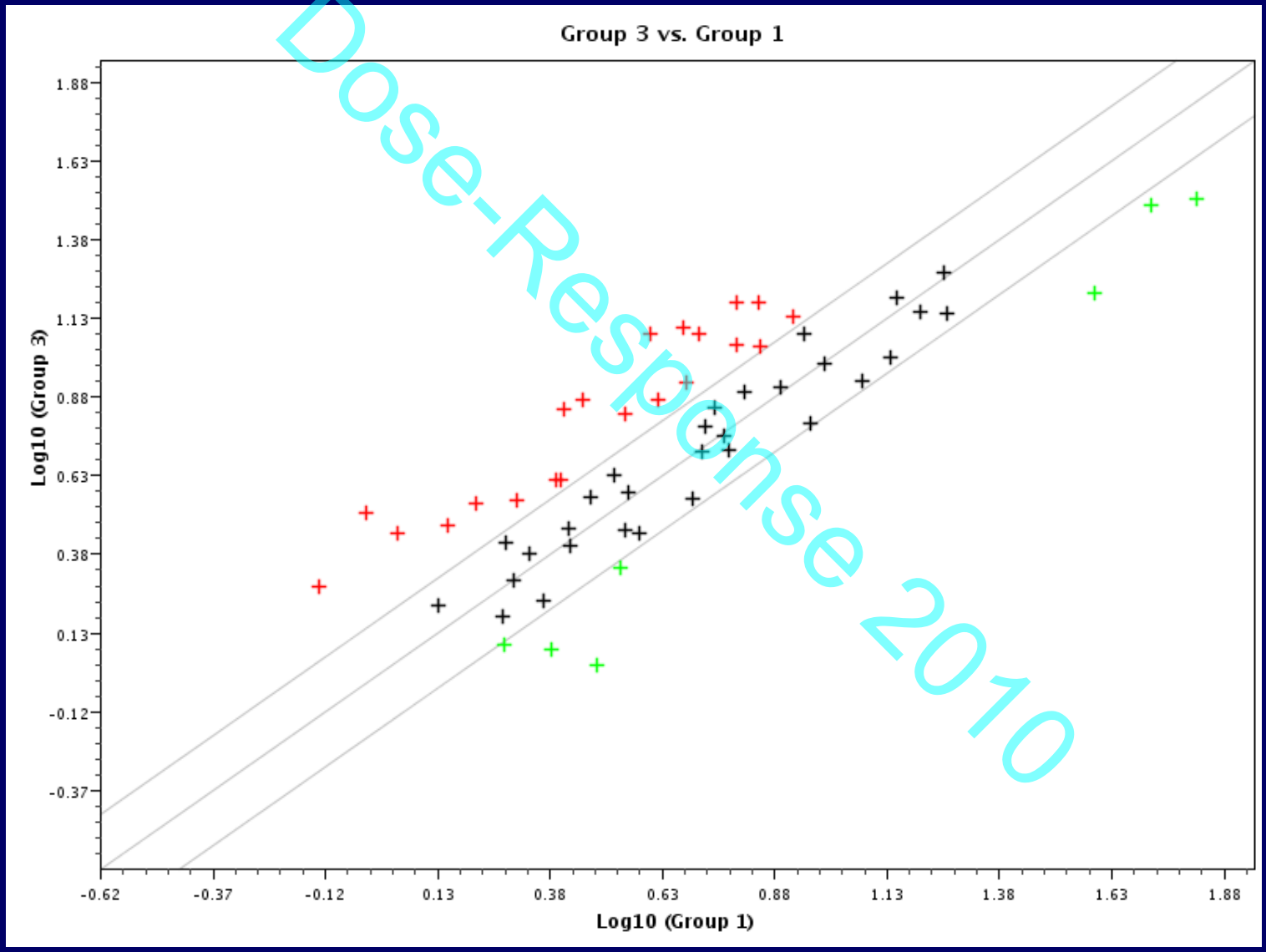
NHEK ECM

NHEK AI

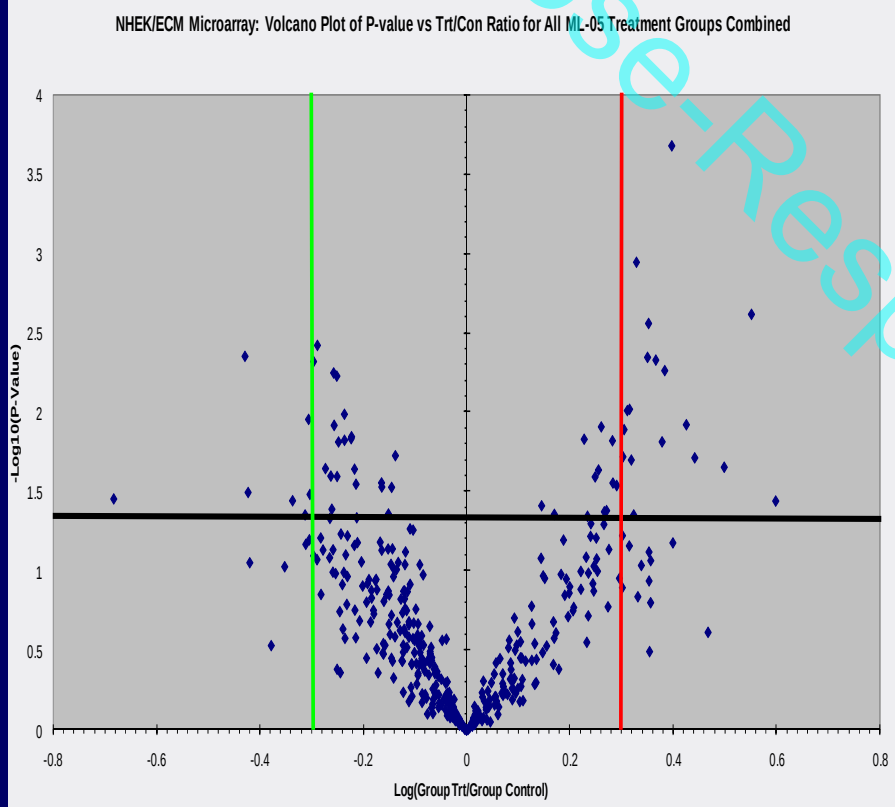
GE Array Analysis Suite: Online Data Analysis

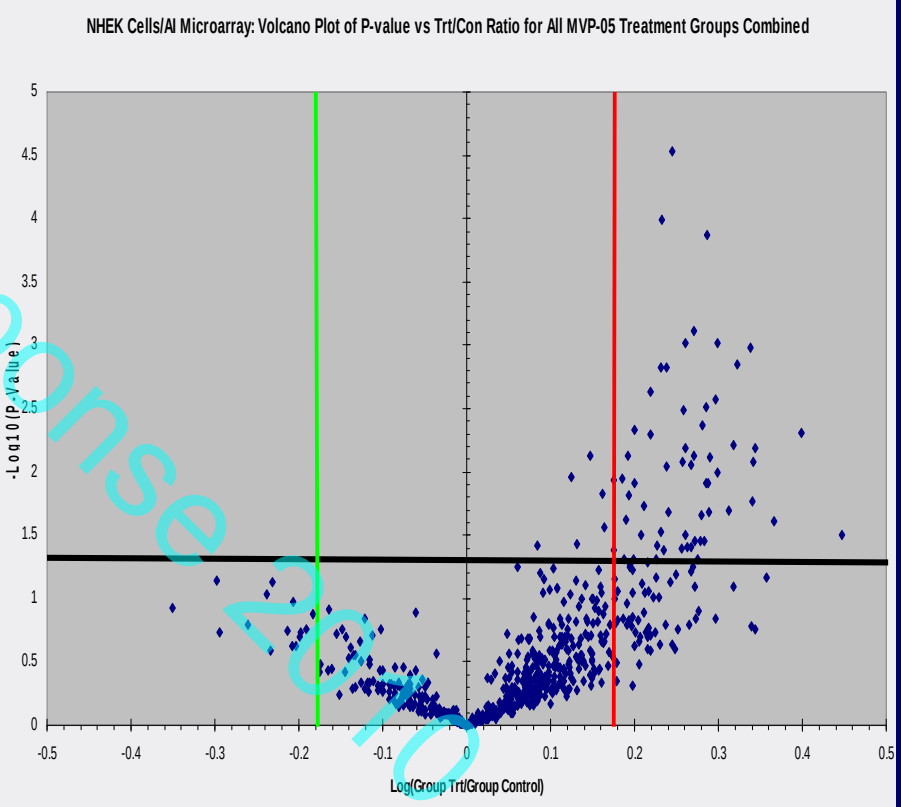
1) Log into Server on Internet
2) Create Project Description
3) Upload Array Images
4) Use Image Data Extraction Application
5) Adjust Image & Acquire Data
6) Analyze Data
7) Export Results to MS Excel
8) Edit and Evaluate (fold change & t-tests)

A Typical Scatter Plot of the Data

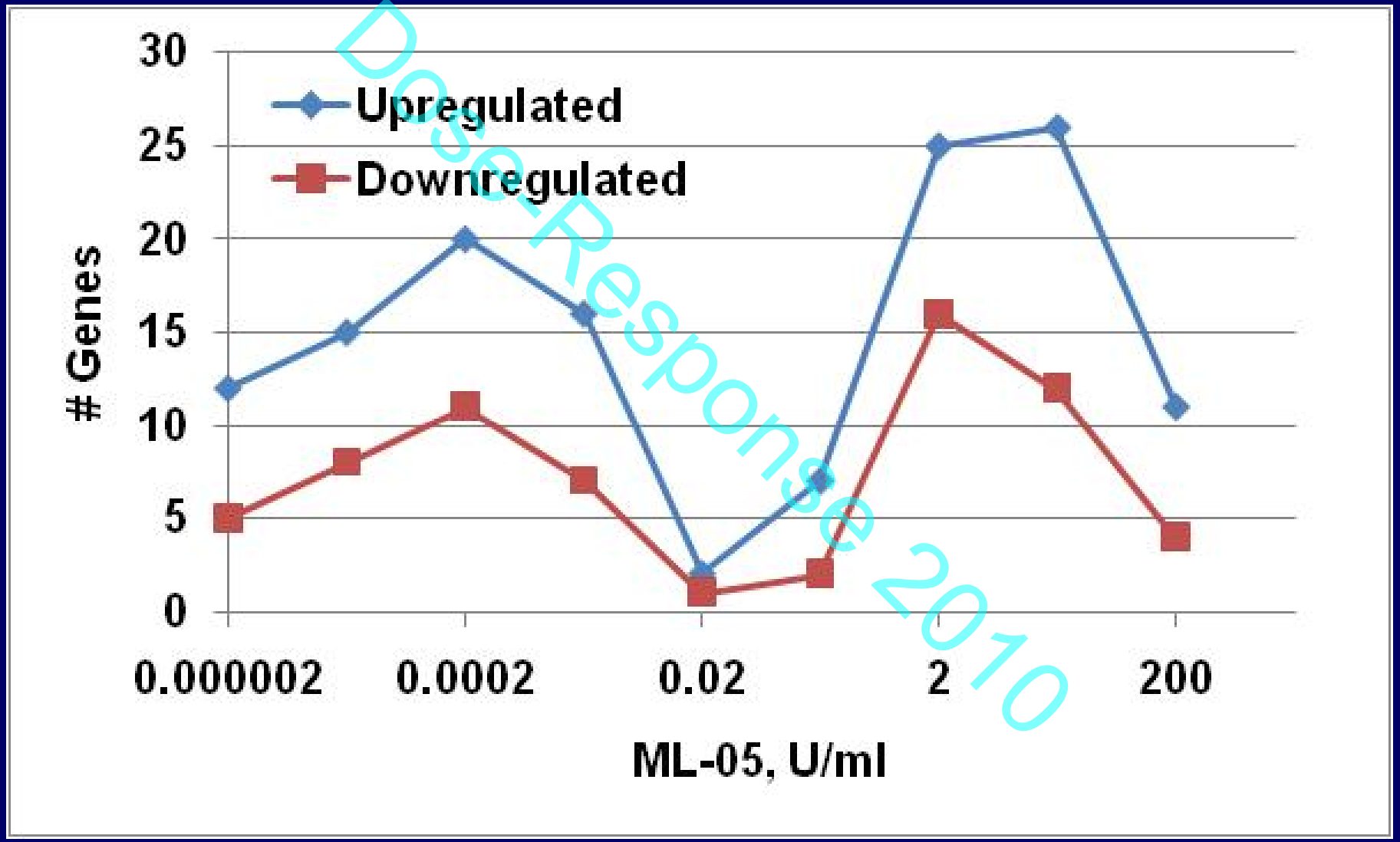


Volcano Plots for ECM and AI Genes (Evaluates Relative Expression and Statistical Significance)





Numbers of Upregulated and Downregulated ECM Genes as a Function of ML-05 Concentration



ML-05 and ECM: Upregulated Genes of Interest Using NHEK Cells (Fold Change >=2, P<0.05)

UniGene	*Symbol*	*Description*
Hs.459142	CD44	CD44 antigen (Indian blood group)
Hs.567249	CNTN1	Contactin 1
Hs.443681	VCAN (CSPG2)	Versican (Chondroitin sulfate proteoglycan 2)
Hs.410037	CTGF	Connective tissue growth factor
Hs.515126	ICAM1	Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor
Hs.553495	ITGA4	Integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)

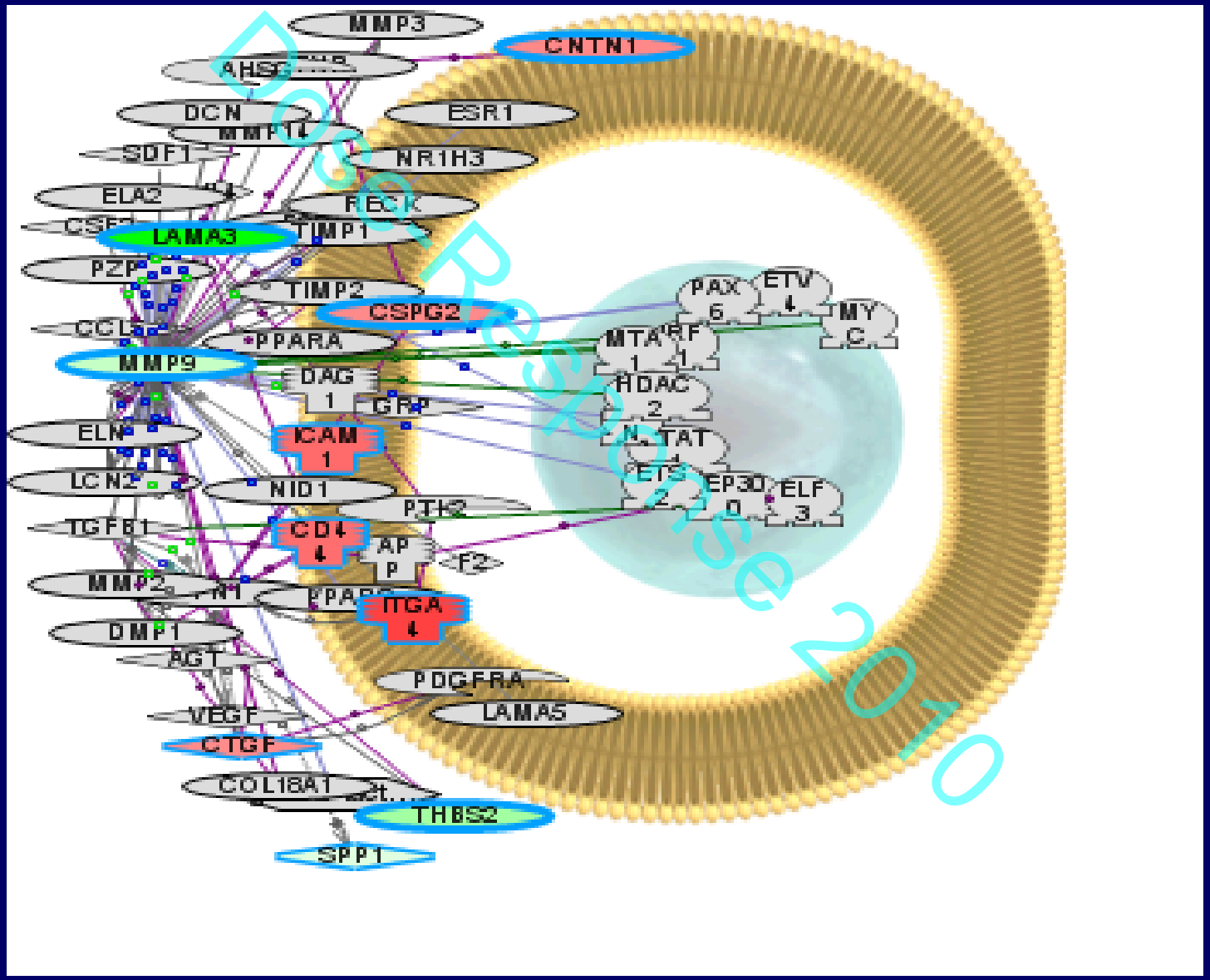
ML-05 and ECM: Downregulated Genes of Interest Using NHEK Cells (Fold Change <=0.5, P<0.05)

UniGene	*Symbol*	*Description*
Hs.436367	LAMA3	Laminin, alpha 3
Hs.297413	MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
Hs.313	SPP1	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)
Hs.371147	THBS2	Thrombospondin 2

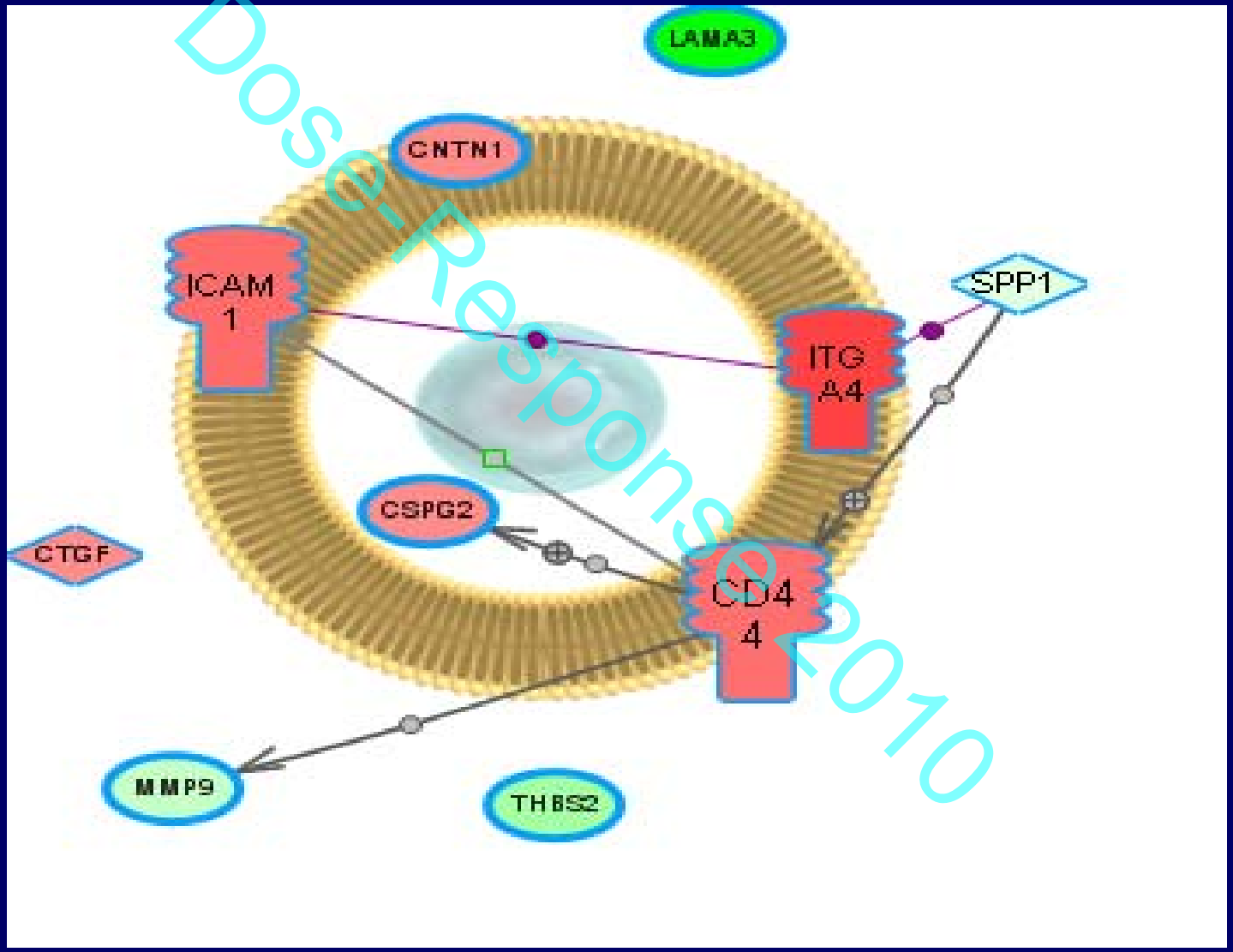
Pathway Analysis Software

In addition to conventional literature searches, the analysis of the gene expression data obtained could benefit from a systems biology/pathway analysis approach to the results. To accomplish this, the gene expression data were analyzed using Pathway Studio (Ariadne Genomics, Rockville, MD). This software encompasses both pathway analysis tools and molecular interaction databases. Depending on the selection of pathway type and filtering criteria used, several different types of pathways can be built from the same original data. This analysis is capable of revealing a number of potential molecular interactions and signaling pathways involving the proteins encoded by the upregulated and downregulated genes products identified in studies such as the current one.

Pathway Analysis – Shortest Path with Extracellular Matrix (ECM) Grouping



Pathway Analysis – Direct Protein Interactions



Polymerase Chain Reaction (PCR) Verification of Genes of Interest (GOI) from the Keratinocyte ML-05/ECM Microarray Studies

1) Conventional PCR Assays - The RT² qPCR Primer Assay is a SYBR® Green-based quantitative real-time PCR assay for gene expression analysis (SABiosciences).

2) Pathway-focused PCR Arrays – New technology from SABiosciences that provides both gene expression profiling and verification of gene regulation in a single platform. This has now replaced the membrane-based DNA microarray (GEArray) system.

ML-05 and ECM: PCR Summary

A. Upregulated Genes of Interest

Symbol	Description	RT-PCR Average	RT-PCR t-test
CD44	CD44 antigen (Indian blood group)	1.13	P<0.5
VCAN (CSPG2)	Versican (Chondroitin sulfate proteoglycan 2)	2.44	P<0.01
CTGF	Connective tissue growth factor	1.46	P<0.01
ICAM1	Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	1.53	P<0.01
ITGA4	Integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	0.77	P<0.01

B. Downregulated Genes of Interest

Symbol	Description	RT-PCR Average Fold Change	RT-PCR t-test
MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	0.57	P<0.01
SPP1	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	0.36	P<0.001

ML-05/NHEK/ECM: Comments on Selected Upregulated Genes of Interest

CD44 - encodes the hyaluronan receptor. This cell surface glycoprotein has a prominent role in cell-to-cell interactions, migration, proliferation and adhesion within the ECM.

VCAN - encodes versican (formerly called CSPG2, for chondroitin sulfate proteoglycan 2). An anti-adhesion molecule, versican has a role in cell migration and proliferation. Both versican and CD44 interact with hyaluronan in maintaining the structural and functional integrity of the ECM.

ICAM1 – encodes intercellular adhesion molecule-1 (CD54). The ICAM1 gene product also can facilitate wound healing by promoting keratinocyte migration.

CTGF - encodes connective tissue growth factor, which functionally resembles tissue growth factor-beta (TGF-beta). This ECM protein also contributes to cell migration and proliferation, and to the production of certain proteins such as integrins. CTGF is capable of modulating the production of both matrix metalloproteinases and tissue inhibitors of metalloproteinases. CTGF has been associated with ECM remodeling in both fibrotic diseases and wound healing.

ML-05/NHEK/ECM: Comments on Selected Downregulated Genes of Interest

MMP9 – Encodes matrix metalloproteinase 9 (AKA gelatinase B), which acts by degrading certain types of proteins within the ECM (gelatin and type IV collagen in particular), as well as receptor proteins at the cell surface. Although some MMPs have a role in tissue remodeling, others have been involved in pathological processes. Moreover, MMP9 can be downregulated through the interaction of CD44 and hyaluronan, and can be modulated by CTGF.

SPP1 - codes for secreted phosphoprotein type 1, commonly known as osteopontin. This glycoprotein component of the ECM is upregulated in inflammatory responses as well as in autoimmune diseases. The downregulation of SPP1 is known to decrease fibrosis and inflammation. SPP1 is also known to be upregulated in several types of cancers.

ML-05 and AI Microarrays: Upregulated Genes of Interest Using NHEK Cells (Fold Change >=1.5, P<0.05)

UniGene	*Symbol*	*Description*
Hs.553486	BDKRB1	Bradykinin receptor B1
Hs.517293	F11R	F11 receptor
Hs.86131	FADD	Fas (TNFRSF6)-associated via death domain
Hs.517240	IFNGR2	Interferon gamma receptor 2 (interferon gamma transducer 1)
Hs.1722	IL1A	Interleukin 1, alpha
Hs.166120	IRF7	Interferon regulatory factor 7

ML-05/NHEK/AI: Comments on Selected Upregulated Genes of Interest

F11R - encodes junctional adhesion molecule-A (JAM-A). This protein is known to be involved in cell migration and in maintenance of epithelial cell morphology.

FADD - encodes Fas-associated death domain, which is involved in inducing apoptosis (programmed cell death). This can be a part of normal homeostatic processes or induced by cell damage or injury.

IFNGR2/IL1A/IRF7 – all encode proteins involved in the cell-mediated immune response. Induction of cell-mediated (or pro-inflammatory) immune responses to SLO have previously been noted in the scientific literature.

These genes have not yet been submitted for PCR-based verification. However, the gene profiling results indicate that ECM gene expression may be more relevant to keratinocyte function than immune response gene expression.

ML-05 Effects on NHDF Gene Expression

Upregulated Genes in HDF Cells (T/C ratios >=2, P<0.05)

UniGene	*Symbol*	*Description*
Hs.420269	COL6A2	Collagen, type VI, alpha 2
Hs.81071	ECM1	Extracellular matrix protein 1
Hs.143250	TNC	Tenascin C (hexabrachion)

Downregulated Genes in HDF Cells (T/C ratios <=0.5, P<0.05)

UniGene	*Symbol*	*Description*
Hs.643357	ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1
Hs.632226	ITGB4	Integrin, beta 4
Hs.536663	ITGB5	Integrin, beta 5
Hs.111779	SPARC	Secreted protein, acidic, cysteine-rich (osteonectin)
Hs.124611	SPOCK1	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 1

Summary

1) Treatment of NHEK cells with ML-05 resulted in the upregulation of four ECM genes (VCAN, CD44, ICAM1 and CTGF) that are directly involved in promoting keratinocyte migration and proliferation.
2) ML-05 also downregulated MMP9 and SPP1 which have potentially detrimental roles in wound healing.
3) ML-05 upregulated the expression of specific immune response genes that may be associated with ECM homeostasis and wound repair.
4) The results provide a mechanistic basis for explaining how ML-05 could promote keratinocyte migration, proliferation and other activities related to ECM organization and wound healing.
5) ML-05 also affected the expression of ECM and immune response genes in NHDF and PBMC cells that may be relevant to the drug's beneficial effects.

Future Research Directions

1) Conversion away from the pathway-focused DNA microarrays (coupled with RT-PCR validation) and validation/implementation of PCR ARRAYS.

2) Complete gene expression/PCR verification studies with normal human dermal fibroblasts and peripheral blood mononuclear cells.

3) Experiments to determine temporal effects of ML-05 on gene expression.

4) Genomics of effects of ML-05 on gene expression in co-cultures of keratinocytes and fibroblasts *in vitro*, or of *in vivo* ML-05-cell/tissue interactions.

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