Gene 550 (2014) 223–229

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Gene expression profiling of *Lucilia sericata* larvae extraction/secretion-treated skin wounds

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ARTICLE INFO

Article history:
Received 11 June 2014
Received in revised form 5 August 2014
Accepted 17 August 2014
Available online 19 August 2014

Keywords:
Lucilia sericata
Larvae
Wound healing
Gene expression

ABSTRACT

The larvae of *Lucilia sericata* have been successfully used as medicinal maggots in the healing of wounds. The excretion/secretion (ES) products of the larvae have been shown to efficiently debride wounds and help the healing process. The mechanisms underlying ES-induced wound healing are not yet completely understood. One of the intriguing questions is the role of ESs in modulating gene expression at the transcriptional level in the skin wound environment during the healing process. To address this question, a study was conducted in which the ES-induced gene expression profile in wound biopsies and ES-treated wounds of rat skin in comparison with control group was analyzed at the molecular level by monitoring the expression of genes associated with wound healing. The expression levels of 82 genes at 4, 7, and 10 days after wounding were determined using a PCR array system following cDNA synthesis. A comparison from wounds revealed that 38 mRNAs (≥5-fold expression) were differentially expressed in the ES-treated skin. For 27 genes, the multiple-test corrected p-value was statistically significant ($p \leq 0.00061$). The expression pattern of these mRNAs was also altered during a period of 10 days. Many of the upregulated or downregulated mRNAs with therapy were extracellular matrix, cell adhesion-related proteins and growth factors. The genes that have the highest fold change (>1000-fold) were *Col1a2*, *Col4a1*, *Ctsk*, *Ccl7*, *Angpt1*, *Cd40lg*, *Egf* and *Itgb5*. Several of these gene products might play key roles in ES-induced wound healing. These findings may provide new insight for an understanding of the therapeutic potential of ESs for wound healing.

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1. Introduction

Maggot debridement therapy (MDT), also called as larval therapy, larvae therapy, biodebridement and biosurgery, has a long history of use in the treatment of chronic and infected wounds. The 1st and 2nd stages of *Lucilia sericata* larvae have been used successfully in the treatment of pathologic conditions such as venous stasis ulcers, decubitus ulcers, temporal mastoiditis, and Fournier's gangrene, and the treatment of necrotic tumor masses and other soft-tissue wounds. Clinical observations and experimental data indicate that the molecules involved in the beneficial effects of maggots are believed to be contained in their excretions/secretions (ESs) (Mumcuoglu et al., 1998; Sherman, 2003; Steenvoorde et al., 2007). The larvae of *L. sericata* help the healing process by the proteolytic digestion of necrotic tissue and disinfection and by the stimulation of granulation tissue formation. In addition, it has been suggested that maggots exhibit other, more direct, mechanisms which contribute to the enhanced healing of wounds (Sherman, 2003; Wollina et al., 2000, 2002). MDT is thus widely used for the treatment of various refractory wounds including diabetic foot and venous ulcers. However, the molecular mechanisms involved in treating non-healing wounds by ESs are not very well understood. Wound healing is a highly complex process involving coordinated interactions between various cellular and biochemical components. Although it has been known that wound healing involves a highly orchestrated multiple-step and events that correlate with the appearance of various cell types in the wound bed during distinct phases of the healing process, many of the limitations in the treatment of pathological condition such as impaired wound healing are based on lack of detailed knowledge of the molecular mechanisms of wound healing.

Presently available evidence indicates that a set of genes is highly regulated at the transcriptional, translational and post translational levels after wound formation (Cooper et al., 2004). The study of the changes in gene expression at the transcriptional level may yield insights into understanding the molecular mechanisms that regulate wound healing and may provide new and more efficient treatment for chronic wounds. Lisa Cooper et al. (2004) revealed that nearly 100

Abbreviations: ESs, excretions/secretions; MDT, maggot debridement therapy; ECM, extracellular matrix.

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http://dx.doi.org/10.1016/j.gene.2014.08.033
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Table 1
Alphabetical sequence of genes investigated and the fold change in mRNA expression in response to the ES-treated wounds at 4, 7, and 10 days.

Gene symbol	Gene description	Day 4		Day 7		Day 10	
		Fold change	p-Value[a]	Fold change	p-Value[a]	Fold change	p-Value[a]
Atct1	Actin, alpha, cardiac muscle 1	−6.06	0.00033[#]	1.77	0.00245	−9.03	0.00002[#]
Angpt1	Angiopoietin 1	−1.24	0.00415	1180.35	0.00066	−2.95	0.00003[#]
Ccl12	Chemokine (C–C motif) ligand 12	1.51	0.00055[#]	3.26	0.00044[#]	1.14	0.00026[#]
Ccl7	Chemokine (C–C motif) ligand 7	−2.92	0.00040[#]	4269.94	0.00118	22.09	0.00333
Cd40lg	Cd40 ligand	1136.20	0.00258	4.81	0.00892	1365.30	0.00040[#]
Cdh1	Cadherin 1	−2.77	0.00173	3.82	0.00253	−2.06	0.00071
Col14a1	Collagen, type XIV, alpha 1	−2.17	0.00095	2142.38	0.00044[#]	−4.68	0.00045[#]
Col1a1	Collagen, type I, alpha 1	−1.02	0.85245	4.89	0.00004[#]	−17.03	0.00195
Col1a2	Collagen, type I, alpha 2	4870.99	0.00234	–	–	−1590.21	0.00262
Col3a1	Collagen, type III, alpha 1	−2.86	0.00288	4.81	0.08961	464.65	0.00085
Col4a1	Collagen, type IV, alpha 1	1530.73	0.00223	4.81	0.07453	149.60	0.00019[#]
Col4a3	Collagen, type IV, alpha 3	–	–	4.81	0.04679	189.36	0.00008[#]
Col5a1	Collagen, type V, alpha 1	−2.76	0.00049[#]	8.22	0.00205	−6.99	0.00058[#]
Col5a2	Collagen, type V, alpha 2	−2.83	0.00033[#]	−1.01	0.78927	−3.28	0.00109
Col5a3	Collagen, type V, alpha 3	1.18	0.24427	−4.10	0.00128	−17.03	0.00007[#]
Csf2	Colony stimulating factor 2 (granulocyte-macrophage)	2.52	0.00004[#]	−2.33	0.00458	−6.02	0.00016[#]
Csf3	Colony stimulating factor 3 (granulocyte)	−2.22	0.00106	−64.22	0.00114	4.00	0.00050[#]
Ctgf	Connective tissue growth factor	−3.90	0.00082	1.11	0.07206	−2.51	0.00331
Ctnnb1	Catenin, beta 1	−2.03	0.00072	−1.16	0.00049[#]	−2.84	0.00024[#]
Ctsg	Cathepsin G	−2.38	0.00295	1.50	0.00004[#]	−2.51	0.00002[#]
Ctsk	Cathepsin K	−10,369.08	0.00059[#]	1.15	0.00141	−1.67	0.00346
Ctsl1	Cathepsin L1	−2.69	0.00190	1.44	0.00038[#]	−2.84	0.00003[#]
Cxcl1	Chemokine (C-X-C motif) ligand 1	1269.46	0.00029[#]	4.81	0.01495	−20.82	0.00013[#]
Cxcl11	Chemokine (C-X-C motif) ligand 11	−2.80	0.00008[#]	−1.21	0.00067	−1.35	0.00421
Cxcl3	Chemokine (C-X-C motif) ligand 3	−1.53	0.00225	−1.19	0.02071	−11.31	0.00002[#]
Cxcl5	Chemokine (C-X-C motif) ligand 5	−2.03	0.00215	−1.11	0.08066	−9.06	0.00014[#]
Egf	Epidermal growth factor	−2.00	0.37386	4.39	0.10003	−10,015.87	0.00178
Egfr	Epidermal growth factor receptor	−2.14	0.00024[#]	1.26	0.00560	−6.96	0.00006[#]
F13a1	Coagulation factor XIII, A1 polypeptide	−3.31	0.00033[#]	1.38	0.00052[#]	–	–
F3	Coagulation factor III	−3.01	0.00146	1.21	0.00024[#]	−2.17	0.00006[#]
Fga	Fibrinogen alpha chain	−3.47	0.00001[#]	1.24	0.00048[#]	−3.42	0.00063
Fgf10	Fibroblast growth factor 10	−2.20	0.00001[#]	1.29	0.00075	−5.03	0.00039[#]
Fgf2	Fibroblast growth factor 2	−6.13	0.00031[#]	1.29	0.00221	−2.59	0.00106
Fgf7	Fibroblast growth factor 7	−1.22	0.00879	−2.29	0.00027[#]	1.21	0.03942
Hbegf	Heparin-binding EGF-like growth factor	−1.71	0.00272	–	–	40.79	0.00061[#]
Hgf	Hepatocyte growth factor	−1.60	0.00265	2.46	0.00027[#]	−8.03	0.00014[#]
Ifng	Interferon gamma	−3.31	0.00052[#]	1.53	0.00033[#]	−7.70	0.00001[#]
Igf1	Insulin-like growth factor 1	−101.13	0.00062	−85.63	0.00040[#]	−3.18	0.00001[#]
Il10	Interleukin 10	−1.89	0.00027[#]	1.36	0.00112	−4.79	0.00003[#]
Il1b	Interleukin 1 beta	−2.62	0.00033[#]	1.41	0.00202	−1.99	0.00078
Il2	Interleukin 2	−9.32	0.00073	1.33	0.00001[#]	−2.00	0.00074
Il4	Interleukin 4	−2.05	0.00058[#]	1.53	0.00001[#]	−1.87	0.00001[#]
Il6	Interleukin 6	−2.37	0.00001[#]	1.39	0.00064	−1.35	0.00021[#]
Il6st	Interleukin 6 signal transducer	−2.93	0.00004[#]	−1.18	0.00063	−1.87	0.00033[#]
Itga1	Integrin, alpha 1	−1.97	0.00086	1.18	0.00001[#]	−3.96	0.00011[#]
Itga2	Integrin, alpha 2	−2.62	0.00017[#]	−2.87	0.00143	−2.06	0.00004[#]
Itga3	Integrin, alpha 3	−14.17	0.00005[#]	−4.16	0.00076	−4.16	0.00005[#]
Itga4	Integrin, alpha 4	1.32	0.00121	1.41	0.00405	−4.58	0.00004[#]
Itga5	Integrin, alpha 5	−1.55	0.01077	−1.04	0.32313	−8.75	0.00018[#]
Itga6	Integrin, alpha 6	−2.54	0.00001[#]	1.31	0.00068	−1.40	0.00085
Itgb1	Integrin, beta 1	−1.20	0.05178	1.46	0.00135	−5.41	0.00056[#]
Itgb3	Integrin, beta 3	−1.25	0.05183	1.31	0.07540	−1.89	0.00017[#]
Itgb5	Integrin, beta 5	−2.31	0.00729	3.66	0.09727	−5752.61	0.00021[#]
Itgb6	Integrin, beta 6	−2.15	0.00047[#]	1.24	0.00146	−2.58	0.00010[#]
Mapk1	Mitogen activated protein kinase 1	−2.31	0.00067	1.60	0.00057[#]	−3.28	0.00009[#]
Mapk3	Mitogen activated protein kinase 3	−12.30	0.00079	1.28	0.00067	−4.20	0.00001[#]
Mif	Macrophage migration inhibitory factor	−16.06	0.00001[#]	1.47	0.00825	−2.01	0.00155
Mmp1a	Matrix metallopeptidase 1a	−2.07	0.00002[#]	1.95	0.00071	−4.87	0.00008[#]
Mmp2	Matrix metallopeptidase 2	−1.89	0.11587	−1.04	0.89322	−45.89	0.00084
Mmp7	Matrix metallopeptidase 7	1.17	0.07235	−261.38	0.00013[#]	−4.03	0.00002[#]
Mmp9	Matrix metallopeptidase 9	−6.80	0.00071	11.47	0.00118	−2.01	0.00100
Pdgfa	Platelet-derived growth factor alpha polypeptide	1.10	0.44791	1.45	0.15367	−11.20	0.00012[#]
Plat	Plasminogen activator, tissue	−1.18	0.00233	1.25	0.00726	−2.68	0.00160
Plau	Plasminogen activator, urokinase	−1.28	0.00224	1.06	0.00135	−3.69	0.00001[#]
Plaur	Plasminogen activator, urokinase receptor	−1.53	0.00038[#]	1.17	0.00167	−3.81	0.00001[#]
Plg	Plasminogen	−2.02	0.00062	1.27	0.00126	−1.14	0.00110
Pten	Phosphatase and tensin homolog	−1.95	0.00035[#]	−1.08	0.00096	−2.77	0.00070
Ptgs2	Prostaglandin-endoperoxide synthase 2	−1.42	0.00088	1.34	0.00351	–	–
Rac1	Ras-related C3 botulinum toxin substrate 1	−3.71	0.00292	1.07	0.03989	−3.47	0.00015[#]
Rhoa	Ras homolog gene family, member A	−1.08	0.00918	–	–	−3.92	0.00175
Serpine1	Serpin peptidase inhibitor, clade E, member 1	−2.65	0.00062	−1.15	0.04696	−2.17	0.00012[#]
Stat3	Signal transducer and activator of transcription 3	−2.81	0.00036[#]	1.82	0.00033[#]	−1.68	0.00001[#]
Tagln	Transgelin	−1.75	0.00125	1.22	0.00006[#]	−2.16	0.00028[#]
Tgfa	Transforming growth factor alpha	−1.50	0.00026[#]	1.95	0.00183	−2.06	0.00172

Table 1 *(continued)*

Gene symbol	Gene description	Day 4		Day 7		Day 10	
		Fold change	p-Value[a]	Fold change	p-Value[a]	Fold change	p-Value[a]
Tgfb1	Transforming growth factor, beta 1	−1.08	0.00048[#]	1.55	0.00054[#]	−4.23	0.00176
Tgfbr3	Transforming growth factor, beta receptor III	−1.43	0.00059[#]	1.23	0.01064	−1.48	0.00040[#]
Timp1	TIMP metallopeptidase inhibitor 1	−1.33	0.00272	1.80	0.00009[#]	−4.16	0.00001[#]
Tnf	Tumor necrosis factor	−1.14	0.00023[#]	1.31	0.00088[#]	−1.91	0.00122
Vegfa	Vascular endothelial growth factor A	−1.33	0.00040[#]	1.22	0.00249	−2.20	0.00074
Vtn	Vitronectin	1.65	0.00001[#]	−1.23	0.07645	−1.63	0.00137
Wisp1	WNT1 inducible signaling pathway protein 1	−1.98	0.00006[#]	1.15	0.00029[#]	−4.41	0.00008[#]
Wnt5a	Wingless-type MMTV integration site family, member 5A	65.34	0.00188	4.81	0.14004	−9.45	0.00001[#]

[a] Uncorrected p value.
[#] p-Values ≤ 0.00061 were considered statistically significant.

genes were expressed with an immediate early gene profile at the wound site by array analysis. Furthermore, several trials on disrupted or delayed and non-healing skin tissues have been performed to identify possible differentially expressed mRNAs (Cha et al., 2008; Seifert et al., 2008; Smith et al., 2008; Wall et al., 2008). These studies reported that many mRNAs were differentially expressed in these tissues than in normally healing skin. On the other hand, few studies have focused on understanding how agents and medicinal plants such as dexpanthenol, *Berberis aristata* DC. (Berberidaceae), *Camellia japonica* L. (Theaceae) and *Panax ginseng* L. (Araliaceae), which are used for the treatment of skin wounds, affect mRNA expression during healing, and whether the altered expression of mRNA may be involved in the skin regeneration (Cho et al., 2009; Heise et al., 2012; Jung et al., 2007; Kim et al., 2008). These investigations revealed the considerable effects of these agents on gene regulation when compared with control samples. However, the role of the ESs of *L. sericata* larvae in modulating gene expression at the transcriptional level in the skin wound environment during healing has not been investigated.

Based on the above considerations, it may be hypothesized that the different expression patterns of mRNAs may be important in inducing the efficient tissue repair of ESs. To address this question, we performed comprehensive mRNA profiling and made a comparative mRNA analysis between ES-induced wound healing tissue and normal healing tissue. Since it has been shown that there are significant alterations in the expression of various genes, with the greatest changes between 3 and 14 days after wounding (Feezor et al., 2004), we followed healing for up to 10 days and analyzed a set of 82 genes which was previously associated with wound healing for 4, 7 and 10 days. Altered expression profiles were detected by facilitating a quantitative reverse transcription real-time PCR (qRT-PCR) system and significant changes were identified by statistical analysis.

2. Materials and Methods

2.1. Animals, Experimental Protocol and Wound Creation

Thirty healthy male Wistar albino rats (weighing 250–300 g, 12 weeks old) were used in this study. All animal studies were performed in accordance with the guidelines of the Istanbul University Experimental Animal Studies Local Ethic Committee. The animals were anesthetized with ketamine (50–100 mg/kg, intraperitoneally) and xylazine (10 mg/kg, intraperitoneally). The dorsal hair was shaved and the skin was sterilized. Full thickness dorsal skin excisions with the size of 1.5 cm in diameter were carried out. The rats were grouped (5 rats/group) as follows: group I — treated with 50 μl *L. sericata* ESs, and group II — treated with Milli-Q ultrapure water (control). Larvae ESs or water was applied topically to the wounds on 0, 4, and 7 days after wounding. Granulation tissues were surgically removed on 4, 7, and 10 days after wounding and stored at −80 °C for molecular genetic analysis. The study protocol was approved by the Animal Research Ethics Committee of Istanbul University, Istanbul, Turkey (November 14, 2011, No: 769).

2.2. Preparation of Maggot Excretions/Secretions

ES was extracted in Milli-Q ultrapure water from sterile second- and third-instar larvae of *L. sericata* from our laboratory. Maggot ESs were collected after incubating approximately 2000 larvae/sterile beaker in 1 ml of Milli-Q ultrapure water for 60 min at ambient temperature. Then, 4 ml distilled water was added at 1 h intervals for 4 h, with the larvae left to deposit their secretions into the water. Next, ES preparations were pooled and centrifuged at 1300 *g* for 5 min at 4 °C to remove particulate material. The equal concentrations of ESs (protein concentration of 50 μg/ml) were used and the animals in group I were treated by ESs of 50 μl per wound.

2.3. Total RNA Extraction and cDNA Synthesis

Total RNA was extracted with Pure link RNA mini kit (Ambion by life Technologies, North America, Cat No. 12183018A) from snap frozen tissue samples following tissue homogenization using MagNa Lyser (Roche Applied Science). Extracted RNA samples were stored at −80 °C until analysis and checked for integrity by running on a 1.5% agarose gel (Sigma Aldrich). RNA quantity and purity were determined by absorbance measurement at 260 nm and 280 nm using the NanoDrop 2000c UV–Vis Spectrophotometer. 1 μg of total RNA was used for cDNA synthesis with reverse aid first strand synthesis kit (Fermentas Life Sciences) according to the manufacturer's instructions.

2.4. PCR Array and Real Time Polymerase Chain Reaction

All amplification reactions were performed using ready to use Rat Wound Healing RT^2 Profiler PCR Array (SA Bioscience) containing primers for 82 tested and 5 housekeeping genes (actin beta, beta-2 microglobulin, hypoxanthine phosphoribosyltransferase 1, lactate dehydrogenase A, ribosomal protein large P1). Included genes are listed in Table 1. The cDNA was diluted, added to the 2× FastStart Universal SYBR Green Master mix (ROX) (Cat. No. 04 913 914 001) according to the manufacturer's instructions and loaded into each well of a 96-well PCR array plate. PCR was performed on a Stratagene MX 3005 Real-Time PCR System (Stratagene, CA). PCR conditions were as follows: initial denaturation at 95 °C for 10 min, and then 40 cycles of denaturation for 1 min at 95 °C, annealing for 1 min at 65 °C and extension for 30 s at 72 °C.

2.5. Data and Statistical Analyses

The data analysis was performed by the RT^2 Profiler PCR Array Data Analysis Template v4.0 (http://www.sabiosciences.com/pcrarraydataanalysis.php). The expression levels in ES-treated wounds relative to controls were analyzed using the $2^{-\Delta\Delta Ct}$ method. The threshold cycle (Ct) was determined for each sample. ΔCt indicated the difference in expression levels with the Ct value of the related gene and mean of five housekeeping genes ($\Delta Ct = Ct_{x\ gene} - Ct_{housekeeping}$), and

ΔΔCt indicated the difference in the ΔCt value between treatment and control groups ($\Delta\Delta Ct = \Delta Ct_{ES} - \Delta Ct_{control}$). The p values are calculated based on a Student's t-test of the replicate $2^{-\Delta Ct}$ values for each gene in the control and treatment groups. A Bonferroni corrected p value of 0.00061 (0.05/82 mRNAs) was applied in statistical tests to compensate the multiple testing error.

3. Results

In this study, we compared the profile of mRNAs associated with wound healing between control and ES-treated wounds. RT^2-PCR array analysis of wound tissues over a course of 10 days of healing showed that 38 out of 82 mRNAs were differentially expressed in the ES-treated wounds to a factor of 5 fold or more in comparison to the control wounds (Table 1). When the multiple testing correction was applied using Bonferroni correction (82/0.05: $p \leq 0.00061$), many genes (27/38) remained statistically significant. The sequence in the table shows the symbol abbreviation (alphabetical order), description of genes, the fold up or downregulation, and the p value.

Comparing with the control wounds on day 4 post wounding, we observed that Cd40 ligand (*Cd40lg*), Collagen, type I, alpha 2 (*Col1a2*), Collagen, type IV, alpha 1 (*Col4a1*), Chemokine (C-X-C motif) ligand 1 (*Cxcl1*), and Wingless-type MMTV integration site family, member 5A (*Wnt5a*) were upregulated. Col1a2 was revealed to have the highest fold change (4870.99-fold) among the 5 upregulated mRNAs. Actin, alpha, cardiac muscle 1 (*Actc1*), Cathepsin K (*Ctsk*), Fibroblast growth factor 2 (*Fgf2*), Insulin-like growth factor 1 (*Igf1*), Interleukin 2 (*Il2*), Integrin, alpha 3 (*Itga3*), Mitogen activated protein kinase 3 (*Mapk3*), Macrophage migration inhibitory factor (*Mif*), and Matrix metallopeptidase 9 (*Mmp9*) were downregulated in the ES-treated wounds. *Ctsk* had the lowest fold change (10,369.08-fold) among the 9 downregulated mRNAs (Figs. 1A, 2A, and 3A). Among genes that were differentially expressed at least 5-fold, Cxcl1, Actc1, Ctsk, Fgf2, Itga3 and Mif were statistically significant when applying the Bonferroni correction (Bonferroni corrected p-value threshold $\leq$0.00061).

In the ES-treated wounds compared to the control wounds on day 7 after wounding, Angiopoietin 1 (*Angpt1*), Chemokine (C–C motif) ligand 7 (*Ccl7*), Collagen, type V, alpha 1 (*Col5a1*), Collagen, type XIV, alpha 1 (*Col14a1*), and Matrix metallopeptidase 9 (*Mmp9*) were upregulated, and Colony stimulating factor 3 (granulocyte) (*Csf3*), Insulin-like growth factor 1 (*Igf1*), and Matrix metallopeptidase 7 (*Mmp7*) were downregulated. *Mmp7* had the lowest expression level (261.38-fold) in the 3 downregulated, and *Ccl7* had the highest expression level with 4269.94 fold change in the 5 upregulated mRNAs (Figs. 1B, 2B, and 3B). After multiple-test correction, a total of 3 out of 8 mRNAs that were differentially expressed at least 5-fold in 7-day old wounds were differentially expressed. The genes that remained significant after multiple-test correction were *Col14a1*, *Igf1* and *Mmp7*.

On day 10 post wounding compared to the control wounds, the genes were activated in the ES-treated wounds, with 6 upregulated and 20 downregulated mRNAs. The upregulated genes were Chemokine (C–C motif) ligand 7 (*Ccl7*), Cd40 ligand (*Cd40lg*), Collagen, type III, alpha 1 (*Col3a1*), Collagen, type IV, alpha 1 (*Col4a1*), Collagen, type IV, alpha 3 (*Col4a3*), and Heparin-binding EGF-like growth factor (*Hbegf*). The downregulated genes were Actin, alpha, cardiac muscle 1 (*Actc1*), Collagen, type I, alpha 1 (*Col1a1*), Collagen, type I, alpha 2 (*Col1a2*), Collagen, type V, alpha 1 (*Col5a1*), Collagen, type V, alpha 3 (*Col5a3*), Colony stimulating factor 2 (granulocyte-macrophage) (*Csf2*), Chemokine (C-X-C motif) ligand 1 (*Cxcl1*), Chemokine (C-X-C motif) ligand 3 (*Cxcl3*), Chemokine (C-X-C motif) ligand 5 (*Cxcl5*), Epidermal growth factor (*Egf*), Epidermal growth factor receptor (*Egfr*), Fibroblast growth factor 10 (*Fgf10*), Hepatocyte growth factor (*Hgf*), Interferon gamma (*Ifng*), Integrin, alpha 5 (*Itga5*), Integrin, beta 1 (*Itgb1*), Integrin, beta 5 (*Itgb5*), Matrix metallopeptidase 2 (*Mmp2*), Platelet-derived growth factor alpha polypeptide (*Pdgfa*), and Wingless-type MMTV integration site family, member 5A (*Wnt5a*). *Cd40lg* was the most upregulated mRNA with 1365.30 fold change, and *Egf* was the most downregulated mRNA with 10,015.87 fold change (Figs. 1C, 2C, and 3C). Among the 26 genes that displayed at least a 5-fold difference in 10-day-old wounds, 20 genes remained significant after Bonferroni correction (Table 1 and Figs. 1C and 3C). The multiple-test corrected p-value of Ccl7, Col3a1, Col1a1, Col1a2, Egf and Mmp2 genes was statistically non-significant, i.e. $p \leq 0.00061$.

4. Discussion

The cutaneous wound healing process is an extremely well-regulated and complex regenerative response to tissue injury among components of the extracellular matrix (ECM), growth factors and cells. It involves changes in the expression of specific mRNA at specific phases of wound healing. Aberrant mRNA expression patterns in orderly and timely manner can prevent the proper healing of wounds. Differences in mRNA expression patterns of the healing-impaired wound have been shown in several studies (Cha et al., 2008; Seifert et al., 2008; Smith et al., 2008; Wall et al., 2008). In the present scenario, we hypothesized that differential expressions of mRNAs in ES-induced wounds might be contributing to accelerated wound healing after ES treatment and the ES-induced gene expression changes in the healing of dermal wounds in rats were investigated. As the molecules responsible for the beneficial actions of maggots are contained in their excretions/secretions (ESs), we assessed the effects of ESs on 82 gene transcripts which are involved in wound-related gene expression by using quantitative RT-PCR array.

When five therapy wounds were compared with five controls, the obtained data showed that many mRNAs were differentially expressed in the ES therapy wounds and that their expression levels varied during a period of 10 days. In this study, we identified 38 potential mRNAs that were differentially expressed by more than five-fold up or down after ES therapy. Many of these mRNAs encode ECM, cell adhesion-related proteins and growth factors.

At day 4 post wounding, we observed upregulation of five mRNA levels involved in ECM organization. Two of them were *Col1a2* ($p = 0.00234$) and *Col4a1* ($p = 0.00223$) which were upregulated by more than 4871- and 1530-fold, but did not reach statistical significance by Bonferroni correction. The *Col1a2* gene, which is among the most induced genes upon ES treatment during the early period of wound healing, encodes the pro-alpha2 chain of type I collagen whose triple helix is comprised of two alpha1 chains and one alpha2 chain. Type I collagen is the most abundant extracellular matrix protein in the skin, which contributes to the stability of the epidermis and is responsible for its tensile strength. Previous studies by Zhang et al. (2011) observed that the mRNA level of COL1A2 is significantly decreased in pathological scar tissues and in normal skin. In addition, it has been shown that several medicinal plants such as *B. aristata* DC. (Berberidaceae), *C. japonica* L. (Theaceae), and *P. ginseng* L. (Araliaceae) which have been reported to have skin aging prevention potential increase the expression of COL1A2 mRNA (Cho et al., 2009; Jung et al., 2007; Kim et al., 2008). As it has been suggested that the improved collagen synthesis is a consequence of early events during the repair process (Kostarnoy et al., 2013), it may be assumed that the high overexpression of *Col1a2* by ES therapy may contribute to a more accelerated wound healing.

The alteration of gene expression by ES treatment for 4 days included the downregulation of *Actc1*, *Ctsk*, *Fgf2*, *Igf1*, *Il-2*, *Itga3*, *Mapk3*, *Mif*, and *Mmp9* mRNA expression (Figs. 1A, 2A, and 3A). The highest downregulation, which is about 10,000-fold, was observed for CTSK mRNA expression after ES therapy. Cathepsin K, a cysteine protease that exhibits strong degradative activity against collagens, elastins and proteoglycans is usually not expressed in healthy skin, while its expression is induced by inflammation or in scar formation (Hou et al., 2001; Quintanilla-Dieck et al., 2009). It has been found in many fibrotic processes including scars, keloids and dermatofibromas (Rünger et al., 2007). Therefore, the suppressed mRNA expression of CTSK in treated wounds may contribute to the delay in scar formation, since CTSK is

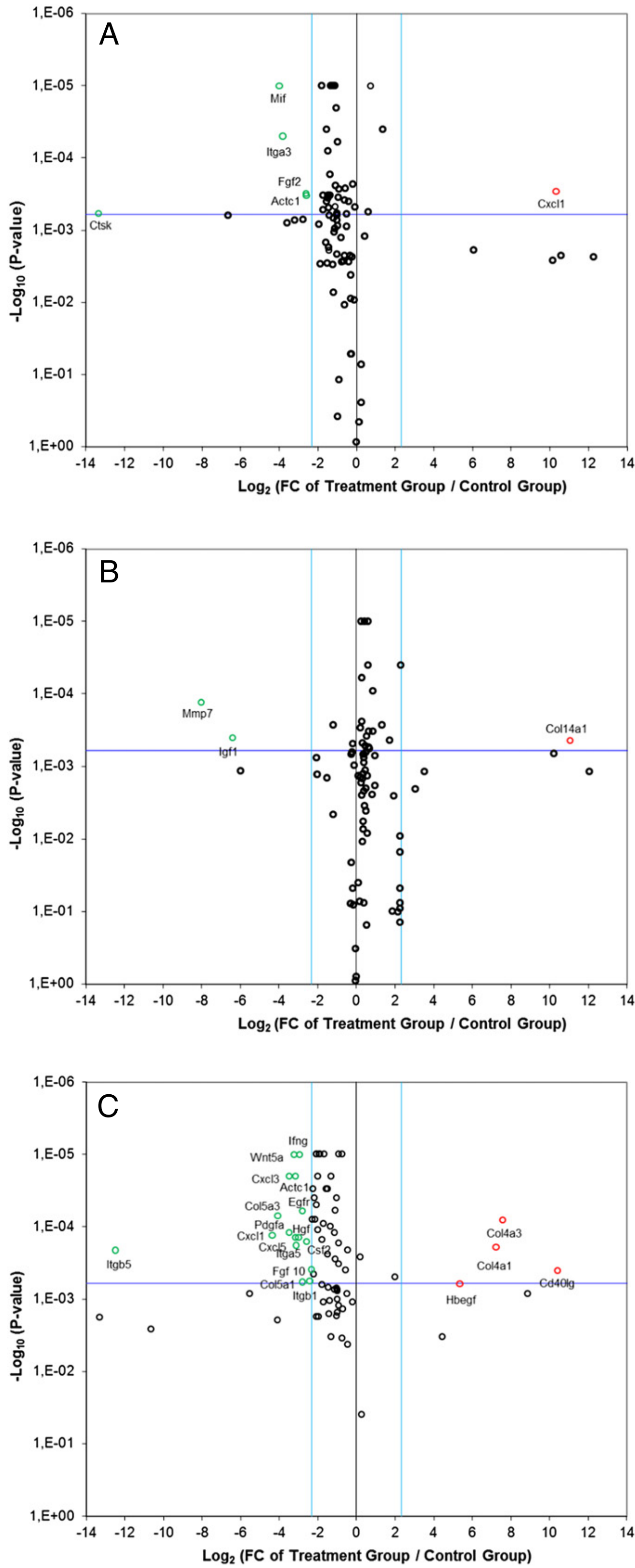

Fig. 1. Scatter plot of the genes associated with wound healing in the ES-treated wounds versus control for 4 (A), 7 (B), and 10 (C) days.

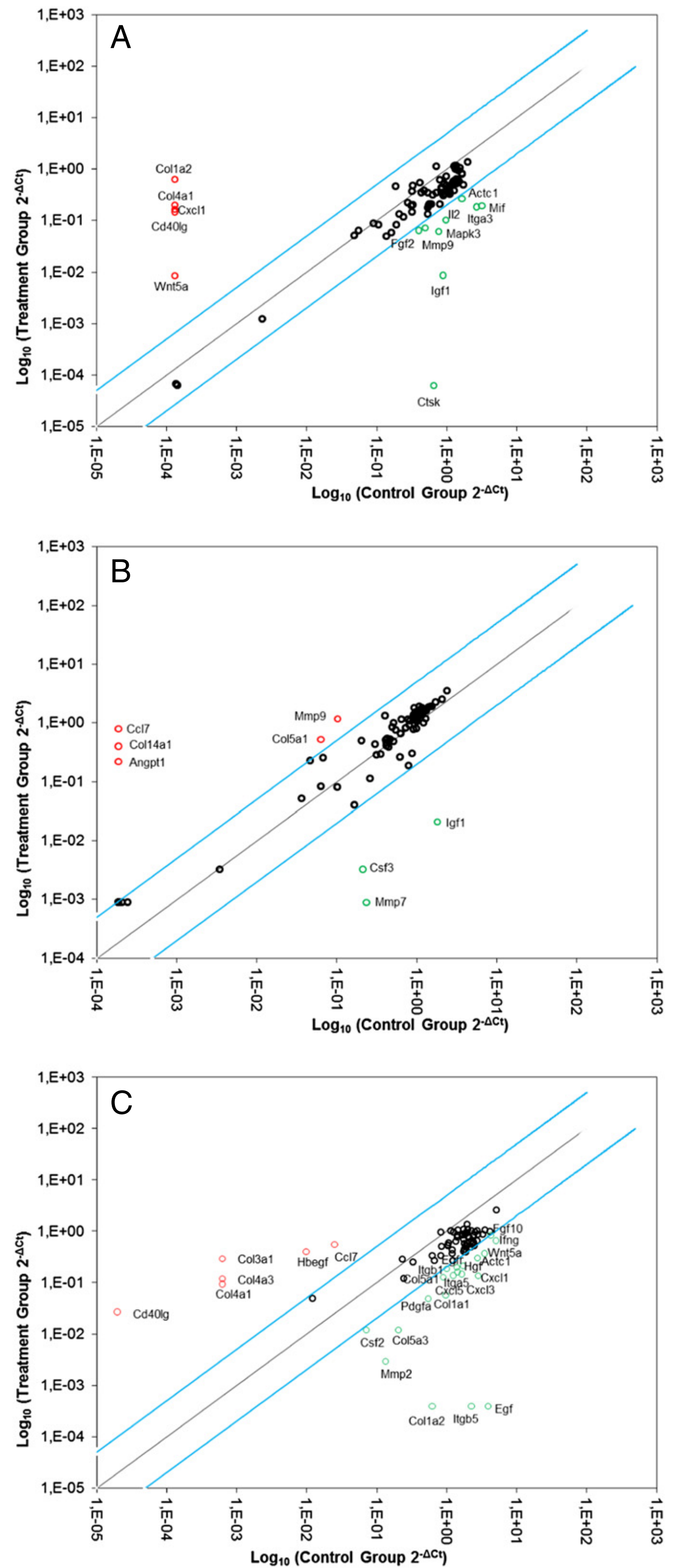

Fig. 2. Volcano plots of the gene expression profiles at each time point. The magnitude of differential expression (log2 fold-change) is plotted versus the level of statistical significance (p-value) of all genes in the PCR-array for (A) 4, (B) 7, and (C) 10 days post-ES exposure.

highly effective at degrading type I collagen (Kafienah et al., 1998). Our histological analysis demonstrated that the application of ES therapy resulted in increased scar free healing (data not shown). Considering the efficient downregulation of *Ctsk* after ES therapy, it may be assumed that ESs may accelerate cutaneous wound repair through suppression of *Ctsk* transcription. However, this question requires further study.

The 5-fold change exclusion showed that 8 genes had altered transcript level in 7-day old ES-treated wounds. In this period, although

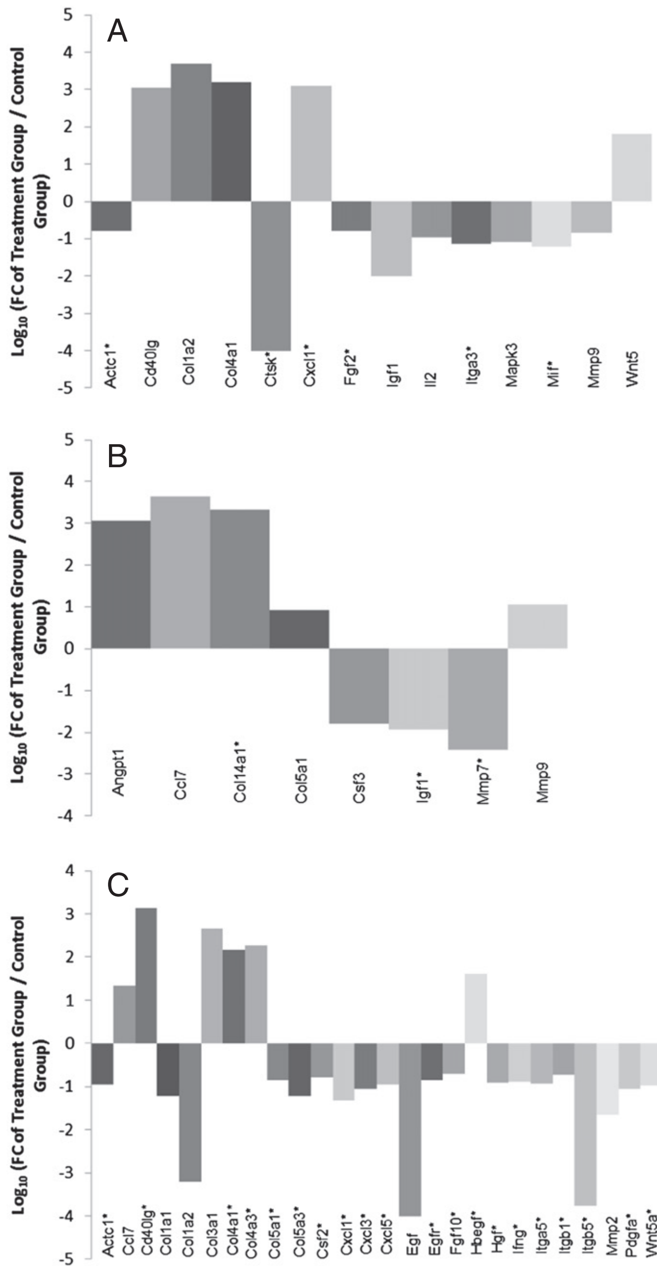

Fig. 3. Magnitude of fold change in mRNA expression in response to ES exposure at (A) 4 days, (B) 7 days, and (C) 10 days. *The multiple-test corrected p-value was statistically significant, i.e. $p \leq 0.00061$.

the genes related with ECM and cell adhesion were remarkably expressed in the ES-treated samples, among others, *Ccl7* and *Mmp7* were the highest up- and down-regulated genes (4270- and 261-fold, respectively). Decreased cytokine and growth factor production including CCL7/MCP-3 has also been shown to play a role in impaired wound healing (Mast and Schultz, 1996; Menke et al., 2007). Chemokine (C–C motif) ligand 7 (CCL7) is a small cytokine known as a chemokine that was previously called monocyte-specific chemokine 3 (MCP3). CCL7/MCP-3 plays a key role in the recruitment of monocytes into the wounded area and promotes monocyte differentiation into macrophages. Macrophages have been shown to play a critical role in wound healing (Gillitzer and Goebeler, 2001). Therefore, it may be hypothesized from our results related to the upregulation of CCL7 transcript expression that ES-stimulated macrophages and possibly other types of cells in the wound microenvironment lead to improved wound healing. Furthermore, we observed that the expression of the angiogenic factor angiopoietin-1 (*Angpt1*) mRNA in ES-treated samples was significantly up-regulated (over 1000-fold) in the day 7 wounds. Following this observation, it may be also speculated that the ES treatment stimulates enhanced angiogenesis, which facilitates healing. Recent evidences indicate an up-regulation of *Mmp7* expression in delayed wound healing (Mäkitalo et al., 2010). There is also sufficient evidence that MMP7 cleaves a number of proteins that may participate in re-epithelialization and wound healing (Ulmer et al., 2010; Wilson and Matrisian, 1996). MMP7, also known as matrilysin-1, is a protease shown to cleave extracellular matrix proteins, and displays a broad proteolytic activity against a variety of extracellular matrix substrates (Wilson and Matrisian, 1996). The findings of the present study show that expression of MMP7 mRNA transcript was lower in ES-treated samples on day 7. The findings of the present study show that expression of MMP7 mRNA transcript was lower in ES-treated samples on day 7. Therefore, our data can provide indirect evidence suggesting that MMP7 suppression can improve wound healing. However, it should be kept in mind that all other genes with altered transcript level, except *Col14a1*, *Igf1* and *Mmp7* did not reach statistical significance after correction for multiple testing. Further investigation is necessary to explain the observed gene expression differences between ES-treated and untreated wounds on day 7.

During the 10 days of healing, one of the most striking findings was the downregulation of many genes with 20 downregulated mRNAs in the ES therapy group. In this phase, we also observed upregulation of 6 mRNA levels, including *Ccl7*, *Cd40lg*, *Col3a1*, *Col4a1*, *Col4a3* and *Hbegf*, although the changes for *Ccl7 and Col3a1* were non-significant when applying the Bonferroni correction. When compared with normal healing, our analysis revealed a sustained increase on days 4 and 10 post-wounding in *Cd40lg* (>1000-fold) throughout the 10 day observation. Cd40lg is a B cell costimulatory molecule, mainly expressed on the surface of activated CD4+ T cells. Considering accelerated wound healing after ES-treatment, and the known role of Cd40lg in T cell differentiation, it can be hypothesized that an increase in Cd40lg expression during the early and late stages of wound healing might be responsible for a better and accelerated immune defense in these terms. On the other hand, in all genes that were down-regulated, the most significant one was the downregulation of the epidermal growth factor (*Egf*) expression. Interestingly, in the present study it was found that there was a tendency towards decreased expression of EGF, nearly 10,000 times lower expression than that of the control group on day 10, although its p-value with correction for multiple testing did not reveal any significant change. The combination between the EGF and its receptor is very important for cellular signal transduction of mitosis (Ballarò et al., 2005). EGF is known as a key promoter associated with proliferation and differentiation of fibroblast and keratinocyte at the wound site to accelerate wound healing (Martin, 1997; Santoro and Gaudino, 2005; Singer and Clark, 1999; Zeigler et al., 1996). Various studies on wound healing have shown that EGF levels increased on day 3, peaked on day 5 and then started to decrease. Although the downregulation of EGF is an expected occurrence during the late period of wound healing, it was found in the present study that ES-treatment strongly suppressed the expression of EGF, demonstrating that ES therapy may produce different effects in the wound microenvironment than those in placebo-treated skin. It is not possible to give an adequate explanation of what causes a decrease in the expression of EGF in the ES-treated wounds during the late period of wound healing, and whether or not this decrease affects the wound healing property of ESs with our experimental design, and the literature published until now. Therefore, additional experiments are necessary to establish the precise mechanism underlying this phenomenon. In addition, it should be noted that there is also a similar uncertainty for the expression of Integrin, beta 5 (*Itgb5*), which is a cell matrix adhesion molecule and which is the most significantly downregulated mRNA (5752.61-fold) in ES-treated wounds on day 10.

The present study necessarily has several limitations. Firstly, we focused on the expression profile of 82 key genes central to the wound

healing response. However, other gene(s), along with the genes investigated in this study, might also contribute to the wound healing property of ESs, taking into consideration that wound healing is a complex process. Another limitation of this study is the lack of quantification of protein level changes associated with our differentially expressed genes. Future studies will be undertaken in order to assess the functionality of the differentially-regulated transcripts with various protein assays. The present study only investigated the changes on the normal healing wound of ES therapy, in the context of gene expression at the transcriptional level; the effects on the gene expression of ES therapy in pathological conditions such as impaired wound healing will be performed in the next experiment. Finally, this study experiment was conducted on a rat model of dermal wound healing and did not involve human tissues.

To summarize, this in vivo rat study revealed the considerable effects of ESs on gene regulation when compared with control treatment in all samples from all three time points of wound healing. Further studies are needed to reveal the importance of the differentially expressed transcripts, and their expression kinetics. Detailed understanding of ES-induced genes will allow us to identify new targets for the treatment of non-healing wounds.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by the Research Fund of the University of Istanbul, project numbers 19654 and 19943.

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