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Determination of effective miRNAs in wound healing in an experimental Rat Model

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Abstract

The larvae of Lucilia sericata have been used for centuries as medicinal maggots in the healing of wounds. The present study aimed to screen potential microRNAs related to ES-induced wound healing in rat skin wounds and to investigate the potential mechanisms contributing to accelerated wound healing. Healthy, male, 12 weeks old Wistar albino rats weighing 250–300 g were supplied by the Animal Experimental Center. All animal studies were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Wistar albino rats were treated by ES after post wounding and the differentially expressed miRNAs in wound biopsies were screened by microarray analysis at the end of treatments for 4,7 and 10 days. In addition, bioinformatics approaches were used to identify the potential target genes of differentially expressed miRNAs and the functions of their target genes. We found a significant up-regulation of rno-miR-99a* and rno-mir-877 in response to ES treatment. Further investigation of rno-miR-99a* and rno-mir-877 and their target genes (TGFa, TNF, TAGLN, MAPK1, MMP-9) implicated in present study could provide new insight for an understanding lead to the development of new treatment strategies. The identified miRNAs can be new biomarkers for ES- induced wound healing.

Key words: microRNA, *Lucilia sericata,* miR-99a*, miR-877, wound healing.

Introduction

Maggot debridement therapy (MDT), also called as larval therapy, larvae therapy, biodebridement and biosurgery, has been employed for centuries in the treatment of chronic and infected wounds. The 1st and 2nd stages of *Lucilia sericata* larvae are still used today in the treatment of pathologic conditions such as various ulcers, temporal mastoiditis, Fournier's gangrene, and the treatment of necrotic tumor masses, decrease smell and alkalinization of the surface area and other soft-tissue wounds. Majority of the experts have believed that the beneficial effects of maggots are created by their active excretions/secretions (ES) (1-3). Wound healing of the damaged tissue is a complex process in which various cell lines including but not limited to those who take part in both acute and chronic inflammation (4, 5). Utilization of maggots from *Lucilia sericata* to clean up the wounded area due to their antibacterial secretions and eating off non-healing necrotic tissue (6- 8). Clinical observations and scientific data suggest that the reasons behind the enhanced effects are due to the maggots' secretions and excretions (ESs) (2, 3). The larva of *Lucilia sericata* improves the healing process by the proteolytic digestion of necrotic tissue, stimulation of granulation tissue formation and disinfecting the wound area (2, 9). Even though it is well known that healing is a highly-regulated multi-step event which its success correlate with the introduction of various cell types throughout the process, most of the limitation in treatment in pathological cases are caused by the lack of detailed knowledge of the molecular mechanisms of these distinct events. Today, we know that several genes are highly regulated at transcriptional, translational and post-translational levels following the wound formation (10, 11).

The aim of this study is to identify and obtain a biomarker based on our understanding of the wound healing process enhanced by larval therapy and further identify molecules taking place in this event. Lisa Cooper et al. revealed that nearly 100 genes were expressed with an immediate early gene profile at the wound site by array analysis (11). Additionally several trials demonstrated that several mRNA are expressed in damaged cells rather than in healthy ones (12-15). In the study, we showed that *Lucilia sericata* larvae ES alter gene expression at the transcriptional level in the cutaneous wound environment during healing process.

These investigations have revealed the potential effects of these agents on gene regulation when compared with control samples. However, roles of the ESs of *Lucilia sericata* larvae are not yet investigated. The healing process is well understood in both tissue and cellular level, although its complex molecular mechanisms are not yet discovered completely. For analyzing and optimizing the healing process, wounds can be described as perturbed molecular networks. It helps to answer specific questions that lead to better understanding of the complexity of the process like: What are the molecular pathways involved in wound healing? How do these pathways interact with each other during the different stages of wound healing? Is it possible to grasp the en-

tire mechanism of regulatory interactions in the process of a wound healing (16)?

MicroRNAs (miRNAs) are endogenously expressed non-coding single stranded RNAs which have approximately 18-22 nucleotides, play key roles in the regulation of target gene expression, most notably in gene silencing (17, 18). MicroRNAs are transcribed in RNA polymerase II as primary transcripts (pri-miRNA) that are cleaved by a protein complex containing the RNase III enzyme DROSHA. They are transported out of the nucleus as a precursor of approximately 70 nucleotide fragments (pre-miRNA) (17, 19, 20).

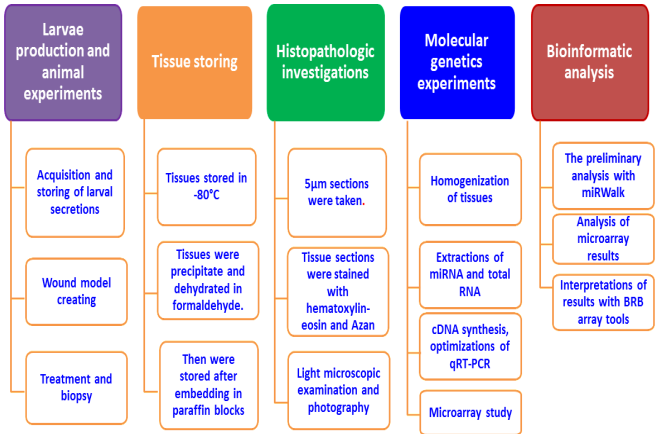
The pre-miRNA is further cleaved by RNAse III "DICER" to form mature single stranded mature miRNAs in the cytoplasm. Mature microRNAs are short, single-stranded RNA molecules (20). Newly formed miRNAs are then joined by RNA-induced silencing complex (RISC) (19). Therefore microRNAs stimulate the gene silencing by depending on their target sites that results in translational suppression of the target mRNA (17, 19).

MicroRNAs have been shown to be involved in a wide range of biological processes such as control of cell cycle, developmental and physiological processes including hypoxia, hematopoiesis, cardiac and skeletal muscle development and immune responses. Besides, in tissue-specific expression pattern during embryogenesis, it is shown that miRNAs play a significant role in the differentiation and maintenance of tissue identity (20).

Based on the above considerations, it can be said that the differential expression of miRNAs may be important in inducing the efficient tissue repair of ESs. Therefore, we performed a miRNA profile screening study and made a comparative mRNA analysis between ES-induced wound healing tissue and normal healing tissue.

Materials and Methods

Healthy, male, 12 weeks old Wistar albino rats weighing 250–300 g were supplied by the Animal Experimental Center. Wistar albino rats were treated in the process of the wound healing. After post wounding and the differentially expressed miRNAs in wound biopsies were screened by microarray analysis at the end of treatments for 4, 7 and 10 days. In addition, bioinformatics approaches were used to identify the potential target genes of differentially expressed miRNAs and the functions of their target genes.



Preparation of Maggot Excretions/Secretions

ES were extracted in Milli-Q ultrapure water from sterile second- and third-instar larvae of Lucilia sericata from our laboratory. Maggot ESs were collected after incubating approximately 2000 larvae/sterile beaker in 1 ml of Milli-Q ultrapure water for 1 hour at ambient temperature. 4 ml distilled water was added at 1 hour intervals for 4 hours, 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.

Experimental Protocol for the creation of acute excision wounds and treatment

Healthy, male, 12 weeks old Wistar albino rats weighing 250–300 g were supplied by the Animal Experimental Center of Cerrahpasa Medical Faculty, Istanbul University and housed for a week before the study in a room with controlled environment (12h light/dark cycle at 23 ± 2°C and relative humidity of 70%). They were also fed by standard laboratory diet and water. All animal studies were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All experiments were approved by the Animal Research Ethics Committee of Istanbul University, Istanbul, Turkey (November 14, 2011, No: 769).

For the creation of acute excision wounds first the animals were anesthetized by an intraperitoneal injection of ketamine (50–100 mg/kg), and xylazine (10 mg/kg). Briefly, the dorsal surface of the rat was shaved and the underlying skin was cleaned with povidone iodine. An acute 1.5 cm diameter circle full thickness excision wound was created by using a scalpel blade at the back of each animal (Figure 1.). All the animals were divided into two groups randomly (n=5 animals in each group). Animals in group I were treated with 50 µl Lucilia sericata ESs, and group II, which was used as negative con-

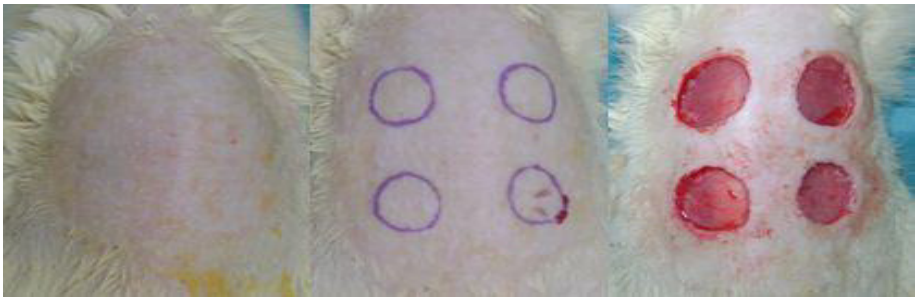


Figure 1. Histology of the Wound. The location of skin wounds at the back of a Wistar albino rat is shown. **(1)** For the array studies a series of criss-cross wounds were made so that all the skin cells were as close as possible to a wound edge for collection of wound tissue for RNA isolation. **(2-3)** For *in situ* hybridization studies and immunohistochemistry a series of four incisional wounds were made.

trol was treated with Milli-Q ultrapure water. 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 (Figure 2.).

The wound diameter was measured and the area (mm^2) within the boundary was calculated planimetrically. The percentage wound contraction was determined using the following formula: percent wound contraction = (original wound area- unhealed area)/original wound area × 100%. Meanwhile the wound tissues were excised to be analyzed by histology, immunohistochemistry, reverse transcriptase polymerase chain reaction (RT-PCR) and microarray experiments.

Histological examination of excised tissue

The excised wound tissue was fixed in 10% neutral buffered formalin, dehydrated in graded ethanol, cleared in xylene, and embedded in paraffin. Five-micron-thick sections were mounted on glass slides and stained with hematoxylin-eosin (HE) and Azan. The sections stained with HE were evaluated for general histology, and the sections stained with Azan were evaluated for epithelial regeneration and fibrosis. Histological slides were investigated with fluorescence microscope Olympus BX40 (Olympus, Japan), using magnification 40–400X. Histological sections were analyzed and interpreted in a blind manner without using any additional information. Pictures of histological specimens were made using a Nikon Digital Sight DS-5M camera (Nikon Corp., Japan). A five-tiered grading system based on degree of re-epithelization, granulation tissue formation and collagen organization was adopted to evaluate the histological differences of different samples (Figure 2.). All subsequent analyses were performed by an experienced pathologist without knowledge of the treatment status.

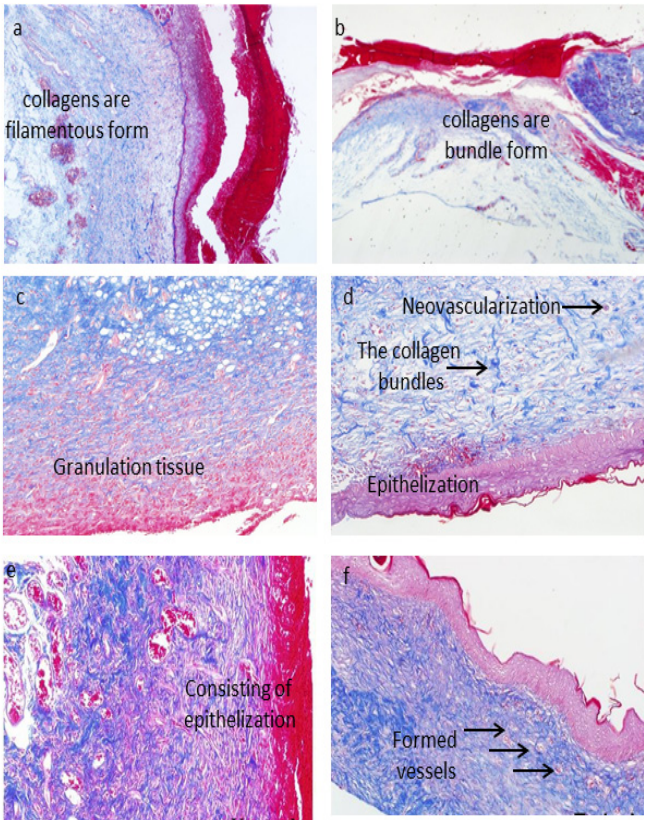


Figure 2. Sections of wound healing. **(a)** Normal group Day-4th **(b)** ES treatment group Day-4 th **(c)** Normal group Day-7 th **(d)** ES treatment group Day-7 th **(e)** Normal group Day-10 th **(f)** ES treatment group Day-10 th

RNA Extraction

Following tissue homogenization using MagNa Lyser beads (Roche Applied Science) microRNAs was extracted from tissue samples using Qiagen miRNeasy Kit (Cat. No 217184; QIAGEN, gMBh, Hilden, Germany) according to the manufacturer's specifications. All RNA samples that were pretreated with RNase-free DNase I (Roche Applied Science, gMBh, Hilden, Germany) to eliminate DNA contamination were quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific). All RNA samples were run on agarose gel to assess their integrity. Samples failing to meet the quality and quantity criteria were either reisolated or completely discarded. Suitable samples were stored at -80°C until microarray analysis.

Microarray Procedure

In this study we used Affymetrix GeneChip® miRNA 2.0 Arrays (Affymetrix, Santa Clara, California, USA) for the analysis of miRNAs in the tissues collected at various time points after wounding of control group versus sacrificed skin, to distinguish key transcriptional events that are part of the tissue repair process independent of inflammatory response.

FlashTag Biotin labeling kit was used according to manufacturer's instructions to label 400 ng of extracted miRNA per sample. Labeling was followed by the addition of poly-A tails. Labeled and poly-A tail added miRNAs were hybridized on GeneChip miRNA 2.0 array (Affymetrix, Santa Clara, California, USA). Arrays with hybridized miRNAs were washed to remove nonspecific binding and stained. Finally, all arrays were scanned to determine miRNA expression levels.

Data and Statistical Analyses

Data analysis, interpretation, and statistical calculations were performed using SPSS software v.17.0 (SPSS Inc., Chicago, IL, USA). Microarray data analysis was performed by Biometric Research Branch (BRB)-Array Tools Version 4.3.2 and t-statistics was performed to obtain differentially expressed miRNAs (30). This is an integrated software system for the comprehensive analysis of microarray experiments. The target genes of the selected miRNAs were performed with miRWalk, which presents predicted and validated information on miRNA-target gene interactions (31). Pathway enrichment analysis was conducted with webgestalt software for pathway analysis (32-34). Principal component analysis (PCA) was performed on all 389 microarray probes with an intensity greater than the background value using the BRB array tools Version: 4.3.0 – Beta-3 and PCA plots were generated with the array tools. For the heatmap clustering of miRNAs with significantly different expression between all sacrificed and control rats, miRNAs were ordered with the clustering complete-linkage method together with the Pearson correlation distance measure. Pearson correlation test was used to analyze the correlations between miRNAs (Figure 3.) with significantly different expression between sacrificed and control rats. The Biological Process Gene On-

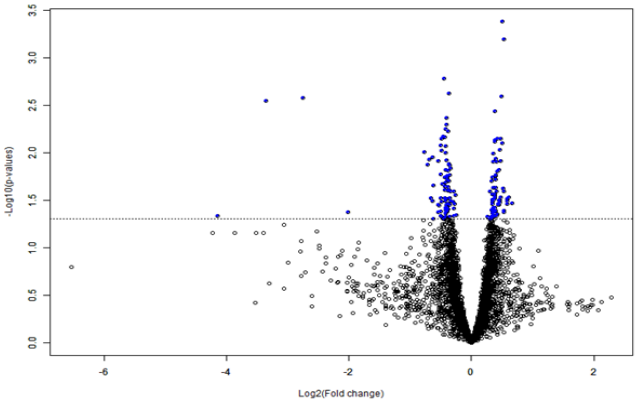


Figure 3. Volcano plot of the gene expression changes in miRNAs. The plot also represents all expressed miRNA genes, including those with less than 1.25 fold changes. Probability values were derived by Student t-test.

tology (GO) was analyzed using the Functional Annotation Clustering tool with the default Rattus norvegicus (Rno) background. The resulting Annotation Clusters were named in a way that best described their GO terms.

Results

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 (21), we followed healing for up to 10 days. We found that 2 miRNAs (rno-miR-99a* and rno-mir-877) had significant change in expression (Table 1.).

We examined the functional trends and regulatory pathways for the miRNAs of the top-hit target genes identified in our study. Biological targets of the genes were predicted using the online web server TargetScan (http://www.targetscan.org/). Three separate softwares were used while pathway analysis. Functional Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, Wikipathway analysis and GO analysis were performed using the online gene set analysis toolkit WebGestalt (http://bioinfo.vanderbilt.edu/webgestalt) (Tables 2-3-4). GO analysis program includes 3 sections, namely the biological process, molecular function, and cellular component.

Target genes of rno-miR-99a* and rno-mir-877 genes were identified and pathway analysis were done. Primarily were subjected pathway analyzed target genes of rno-miR-99a* and rno-mir-877. Subsequently were subjected pathway analyzed to common target genes of rno-miR-99a* and rno-mir-877. Finally were subjected pathway analyzed to common target genes of rno-miR-99a* and rno-mir-877 with in wound healing pathways panel (10) together (Figure 4.).

The KEGG pathway analysis of the target gene for

Table 1. The expression levels of miRNAs that biomarker candidates.

Control vs Treatment	Fold change	ES treatment	Control	P value
rno-miR877-st	1.31	2.55	1.94	0.017
rno-miR-99a*	1.25	2.22	1.77	0.040

Table 2. KEGG pathway, GO Categories and wikipathway analysis for targeted genes of rno-miR-99a* and rno-miR-877.

KEGG Pathway		GO Categories						Wikipathway	
	No. of Genes	Biological process	No. of Genes	Molecular function	No. of Genes	Cellular component	No. of Genes		No. of Genes
(A) rno-miR-99*									
MAPK signaling pathway	30	Establishment of localization	234	Amide binding	24	Cell part	653	MAPK signaling pathway	21
Endocytosis	28	Regulation of biological quality	191	Ion binding	260	Cell periphery	246	G protein signaling pathways	13
Toxoplasmosis	19	Regulation of endogenous stimulus	115	Protein binding	359	Plasma membrane	238	IL-2 signaling pathways	13
Insulin signaling pathway	16	Response to organic substance	176	Receptor binding	89	Cytoplasmic part	363	Insulin signaling	20
Cytokine-cytokine receptor interaction	22	Regulation of transport	107	Protein complex binding	42	Synapse part	56		
Chagas Disease (American trypanosomiasis)	17	Transport	234	Anion binding	137	Intracellular part	575		
(B) rno-mir-877									
MAPK signaling pathway	18	Regulation of transport	120	Substrate-specific transmembrane transporter activity	112	Cell projection part	89	MAPK signaling pathway	17
Cytokine-cytokine receptor interaction	26	Establishment of localization	305	Transmembrane transporter activity	117	Axon	60	IL-3 signaling pathway	15
Cell adhesion molecules (CAM)	19	Response to stress	217	Inorganic cation transmembrane transporter activity	68	Plasma membrane	290	Insulin signaling	18
Vascular smooth muscle contraction	16	Response to organic substance	185	Metal ion transmembrane transporter activity	63	Plasma membrane part	147	Adipogenesis	15

KEGG=Kyoto Encyclopedia of Genes and Genomes, MAPK=Mitogen-Activated Protein Kinase, GO=Gene Ontology.

Table 3. KEGG pathway, GO Categories and wikipathway analysis for common genes of rno-miR-99* and rno-miR-877.

KEGG Pathway		GO Categories					Wikipathway	
	No. of Genes	Biological process	No. of Genes	Molecular function	No. of Genes	Cellular component	No. of Genes	No. of Genes
Cytokine-cytokine receptor interaction	8	Establishment of localization	70	Substrate-spesific channel activity	14	Cytoplasm	130	MAPK signaling pathway	6
Long-term depression	5	Response to alkaloid	11	Cation channel activity	12	Plasma membrane	79	Oxidative stress	3
Neurotrophin signaling pathway	7	Regulation of localization	42	Channel activity	16	Axon	16	Insulin signaling	9

KEGG=Kyoto Encyclopedia of Genes and Genomes, MAPK=Mitogen-Activated Protein Kinase, GO=Gene Ontology.

Table 4. KEGG pathway, GO Categories and wikipathway analysis for common target genes of rno-miR-99* and rno-miR-877.

KEGG Pathway		GO Categories					Wikipathway	
	No. of Genes	Biological process	No. of Genes	Molecular function	No. of Genes	Cellular component	No. of Genes	No. of Genes
Leishmaniasis	2	Response to molecule of bacterial origin	3	Protein binding	5	Plasma membrane	3	EBV LMP1 signaling	2
Type II diabetes mellitus	2	Response to nicotine	2	Catalytic activity	3	Integral to plasma membrane	2	Matrix Metal-loproteinases	2
NOD-like receptor signaling pathway	2	Epitelial cell proliferation	3	Phosphotrans-ferase Activity, alcohol group as acceptor	2	Extracellular region part	3	MAPK signaling pathway	2
Fc epsilon RI signaling pathway	2	Positive regulation of mitosis	2	Protein kinase activity	2	Extracellular space	3	IL-3 signaling pathway	2

KEGG=Kyoto Encyclopedia of Genes and Genomes, MAPK=Mitogen-Activated Protein Kinase, GO=Gene Ontology.

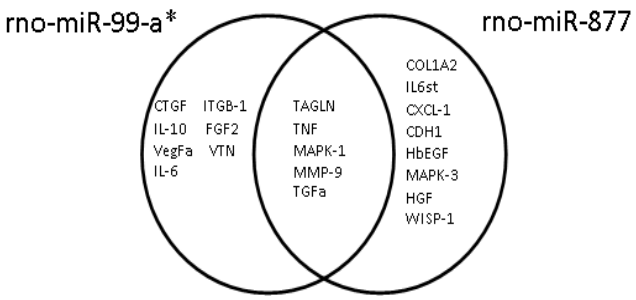


Figure 4. Venn diagram of common target genes predicted for rno-miR-99a* and rno-miR-877.

rno-miR-99* indicates that 30 genes (10.5%) are involved in mitogen-activated protein kinase (MAPK) signaling pathway and 16 (5.6%) in insulin signaling pathway, 22 genes (7.7%) in Cytokine-cytokine receptor interaction. The wikipathway analysis of the target gene rno-miR-99* indicates that 21 genes (13.6%) are involved in MAPK signaling pathway, 20 genes (12.9%) in insulin signaling pathway, 13 genes (7.7%) in IL-2 signaling pathway, 13 genes (7.7%) G protein signaling pathway (Table 2.).

Eighteen target genes (9.2%) for rno-miR-877 are involved in MAPK pathway, 26 genes (8.5%) in Cytokine-cytokine receptor interaction, 19 genes (6.2%) in Cell adhesion molecules (CAM) and 16 genes (5.2%) in Vascular smooth muscle contraction. The wikipathway analysis of the target gene rno-miR-877 indicates that 17 genes (9.6%) are involved in MAPK signaling pathway, 18 genes (10.1%) in insulin signaling pathway, 15 genes (8.4%) in IL-3 signaling pathway, 21 genes (11.8%) Tumor Growth Factor (TGF)-beta receptor signaling pathway, 15 genes (8.4%) in Adipogenesis, 14 genes (7.9%) in Senescence and autophagy and 13 genes (7.3%) in Peptide GPCRs (Table 2.).

The GO analysis of the biological process of the target gene for rno-miR-99a* indicates that 234 (13.9%) of the genes are involved in Establishment of localization and 107 (6.3%) Regulation of transport. Three hundred five (14.2%) of the target gene for rno-miR-877 are involved in Establishment of localization, 120 (5.5%) Regulation of transport, and 185 (8.69%) in Response to organic substance (Table 2.).

We also attempted to predict the potential rno-miR-99a* and rno-miR-877 targets. After comparing the target predictions from the three different databases, the number of overlapping genes was 12 for miR-99a* (TGFa, Connective Tissue Growth Factor (CTGF), Vascular Endothelial Growth Factor (VEGFa), Inter-Leukin10 (IL10), Tumor Necrosis Factor (TNF), Fibroblast Growth Factor2 (FGF2), Vitronectin (VTN), Integrinsubunit beta1 (ITGB1), Transgelin (TAGLN), MAPK1, Matrix Metallo Peptidase-9 (MMP-9) and IL6) and 13 for miR-877 (TAGLN, TGFA, Collagen type I alpha2 (COL1A2), TNF, IL6ST, Chemokine (C-X-C motif) ligand11 (CXCL11), Cadherin1 (CDH1), MMP-9, HbEGF, MAPK3, WNT1 inducible signaling pathway protein1 (WISP1), Hepatocyte Growth Factor (HGF) and MAPK1). In addition, TGFa, TNF, TAGLN, MAPK1 and MMP-9 are predicted to be common target genes of both miRNAs.

Discussion

There is a consensus among experts that the regulation of wound healing by the molecular processes and pathways is a highly orchestrated multiple-step and events. It has been shown that miRNAs levels are altered during normal skin wound healing and in pathological stages leading to wound healing defects, such as

diabetes (22, 23). Therefore, it is reasonable to expect that the study of the changes in ES-induced miRNA expression at wound microenvironment may yield insights into understanding the molecular mechanisms that regulate wound healing and may provide new and more efficient treatment strategies for chronic wounds. Therefore, to reveal whether the different expression patterns of miRNAs may be important in inducing the efficient tissue repair of ES, we compared the miRNA expression profiles of the ES-treated wounds and control wounds from rats. With the use of miRNA microarray, which is a high-throughput method of detecting thousands of miRNAs simultaneously, it was identified that 54 miRNAs were differentially expressed between ES-induced wound healing tissue and normal healing tissue during a period of 10 days. Thirty of them were up-regulated in ES-induced wounds and the remaining twenty-four miRNA were down-regulated. In them, BRB-Array Tools filtering revealed that rno-miR-99a* and rno-mir-877 were up regulated in ES-treated wound of rats. An interesting observation is that the increased expression of both miRNAs was seen over a course of 10 days of healing.

To date, nearly nothing is known about the role of rno-miR-99a* in the wound healing. MicroRNA-99 family, including miR-99a has been shown to contribute to wound healing by regulating the AKT/mTOR signaling and homeobox A1 expression (24). Furthermore, it has been shown *in vitro* that ectopic transfection of miR-99 family to epithelial cells led to reduced proliferation and cell migration, as well as enhanced apoptosis (24, 25). From these observations, we can conclude that the difference in rno-miR-99a* expression may contribute to the regulation of major process influencing wound healing such as cell proliferation, migration, and apoptosis. However, using existing literature data, it would be hard to explain the role of miR-99a in context to signaling pathways and cell proliferation in overlapping phases of wound healing.

In our study, rno-mir-877, which is another top-hit miRNA is highly expressed during wound healing in rat skin tissues treated with ES. Just few information is available for the biological function of miR-877. It was reported that miR-877 could inhibit hepatocellular carcinoma cell proliferation though targeting ForkheadboxM1 (FOXM1) *in vitro* (26). If we simply consider this data as associated with cell proliferation which is one from coordinated events of the wound healing, it may be predictable that that elevated expression of mir-877 by ES exposure may play a role in the accelerated original tissue regeneration by blocking specific mRNAs involved in cell proliferation. However, there is a need to perform more experimental research to and to prove this hypothesis and clarify the biological function of miR-877.

To understand the functional significance of two miRNAs identified in this study and related biological networks in wound healing, the computational predictions of the miRNA target genes was needed. Therefore, we performed the bioinformatics analysis of target genes by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, GO Categories and wikipathway analysis, having different predictive algorithms. Firstly, the targets for rno-miR-99a* and rno-miR-877 were separately analyzed, since each miRNA potentially may regulate many targets. In this case, two changed miRNAs in this study are involved in numerous biological functions or pathways. Rno-miR-99a* was associated with many signaling pathways, including MAPK, insulin signaling, IL-2 signaling, and G protein signaling. It is not surprising that multiple signaling pathways are associated with restoring of effective wound healing. It had already been known that these pathways by directing cellular responses to various stimuli play distinct roles in cell activation, proliferation, differentiation, and survival involved in multiple steps of the wound-healing process. Similarly, network analysis revealed that the majority from target genes of rno-miR-877 was also related to cell signaling and kinase activity. These two observations may imply that both miRNAs in ES-induced wound healing might be contributing to effective tissue repair by influencing multiple signaling pathways. These findings also strengthened the notion that the rno-miR-99a* and rno-miR-877 are key participants in regulation of signal pathways in context to ES-treatment at wound microenvironment.

It has been known that skin wound healing is characterized by effectively regulation of the intrinsic apoptotic pathways and apoptotic cells may be important role in determining the fate of wound healing. Increased apoptosis resistance is also shown to influence scarring and fibrosis (27). The current study demonstrated that apoptosis-related genes (15 genes) were found to be targeted by rno-miR-99a*. On the other hand, in our network diagram, an interesting point is that rno-miR-877 is predicted to target several genes including adipogenesis, senescence and autophagy. Increasing evidence indicates that this process may be an important role in inducing the efficient tissue repair. There is also the idea that pro-senescent therapies contribute to minimize the damage by limiting proliferation and fibrosis during active tissue repair.

Secondly, we sought to further reduce the putative targets by the combination of rno-miR-99a* together with rno-miR-877. They cooperatively target a network of genes involved in various channel activities and transporter activities, which is necessary for effective tissue repair. In this case, regulation of biological quality, localization and transport, and ion and organic substance transport were frequently affected GO terms. All these processes have to be precisely regulated; otherwise interference in the expression of genes related to these events can induce undesirable effects in the damaged tissue and extreme defects in healing, such as tissue fibrosis and prevention of regenerative mechanisms.

Finally, we aimed to predict the target genes of rno-miR-99a* and rno-miR-877. The bioinformatics analysis indicated that common target genes of both miRNAs were TGFa, TNF, TAGLN, MAPK1 and MMP-9. Interestingly, all of these transcripts in ES-treated wounds were the significantly downregulated mRNAs at days 4 and 10 post wounding, while they were significantly upregulated at day 7 in study of Polat et al. (10). In general, it is known that increased miRNA expression causes the inhibition of specific mRNA expression, but there are several reports in the literature that it can also facilitate target mRNA expression (28, 29). ES could

first induce the expression of rno-miR-99a* and rno-mir-877, resulting in the downregulation of TGFa, TNF, TAGLN, MAPK1 and MMP-9 on especially days 4 and 10 post-wounding, which in turn induces the efficient tissue repair. Validation of these mRNA-miRNA target interactions in the ES-treated wounds and quantification of the altered protein levels associated with the predicted genes would allow us to identify new targets for the treatment of non-healing wounds.

In conclusion, this study demonstrated that ES, used to efficiently debride wounds and help the healing process, alters microRNA expression during the course of wound healing of 10 days in rat skin. Our results showed that two miRNAs rno-miR-99a* and rno-mir-877 were induced in rat excisional wounds after exposure to ES. Their target genes are involved in a variety of biological functions, particularly cell signaling, regulation of biological quality, localization and transport, and ion and organic substance transport. Integrated mRNA–miRNA bioinformatics analyses (TGFa, TNF, TAGLN, MAPK1 and MMP-9) are predicted to be common target genes of both miRNAs. The identified miRNAs and the predicted genes in this study, if confirmed by future studies, could be new biomarkers for ES- induced wound healing, and could prompt novel perspectives for better understanding.

Acknowledgements

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