Biogerontology (2016) 17:763–770
DOI 10.1007/s10522-016-9650-z

RESEARCH ARTICLE

Middle age has a significant impact on gene expression during skin wound healing in male mice

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Received: 25 March 2016 / Accepted: 13 May 2016 / Published online: 30 May 2016

Abstract The vast majority of research on the impact of age on skin wound healing (WH) compares old animals to young ones. The middle age is often ignored in biogerontological research despite the fact that many functions that decline in an age-dependent manner have starting points in mid-life. With this in mind, we examined gene expression patterns during skin WH in late middle-aged versus young adult male mice, using the head and back punch models. The rationale behind this study was that the impact of age would first be detectable at the transcriptional level. We pinpointed several pathways which were over-activated in the middle-aged mice, both in the intact skin and during WH. Among them were various metabolic, immune-inflammatory and growth-promoting pathways. These transcriptional changes were much more pronounced in the head than in the back. In summary, the middle age has a significant impact on gene expression in intact and healing skin. It seems that the head punch model is more sensitive to the effect of age than the back model, and we suggest that it should be more widely applied in aging research on wound healing.

Keywords Wound healing · Skin · Mice · Aging · Middle-age

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Electronic supplementary material The online version of this article (doi:10.1007/s10522-016-9650-z) contains supplementary material, which is available to authorized users.

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Introduction

Tissue repair (often referred to as wound healing) is a fundamental process in all multicellular organisms. Some species from diverse taxa (such as hydra, axolotl, salamander, and several others; Gardiner 2005) and early mammalian embryos are able to fully regenerate damaged tissues/organs (Ferguson and O'Kane 2004). In mammals, this ability is drastically reduced after birth and continues to decline with age. This reduced regenerative capacity is in fact a desired response, where speed of wound healing (WH) is favored over functional restoration, so that the outcome is the formation of scar tissue (Ferguson and O'Kane 2004). Deviations from regular repair may lead to diverse pathological conditions, from slow or ineffective WH to excessive fibroproliferative responses (Grose and Werner 2003), both of which are often observed in advanced ages.

There is a consensus that skin healing in the elderly is delayed but the final result is qualitatively similar to that in young subjects (reviewed by Gosain and DiPietro 2004). In fact, the rate of skin WH is often used as a marker of biological age (Yanai et al. 2011, and references therein).

The vast majority of research on the impact of age on skin wound healing compared old animals to young ones. The middle age is often ignored in biogerontological research despite the fact that many functions that decline in an age-dependent manner and the development of age-related diseases, have starting points in mid-life (Hekimi 2006; Wang et al. 2007; Rattan 2015). In other words, the state of a given function in the middle-age may to a great extent determine the state of that function in the old age.

It is reasonable to assume that processes that have already begun but are not yet fully manifested, would first be detectable at the transcriptional level. With this in mind, we focused on gene expression patterns during skin wound healing in late middle-aged versus young mice.

Materials and methods

Animals

Following ethical board review (Medical University of Vienna) the Austrian Ministry for Science and Research (BMWF 3568-2008/09-115-1997/98) approved the conduction of experiments. Male C57/BL6 J mice were commercially obtained from Charles River Laboratories (Sulzfeld, Germany) and maintained at the Department for Biomedical Research, Medical University of Vienna, according to the Austrian Animal Experimental Research Act and European Union Guideline 2010/63/EU for the care and use of laboratory animals. The experiments were carried out on male mice of two age groups: young adult (3 to 4-month-old) and late middle-aged (17 to 18-month-old). Overall, 26 mice (12 young adult and 14 middle-aged) were experimented on. Only animals with no overt pathological manifestations as confirmed by autopsy examination were included in the experiments. To minimize the influence of circadian variation, surgery, tissue collection and digital photography of the wounds were performed during the light hours between 10:00–12:00.

Head and back excision model

Mice were intra-peritoneally anesthetized before surgery with xylazine 5 mg/kg body weight and ketamine 100 mg/kg body weight. Full-thickness wounds were initially marked on the crown of the skull or the back midsection, using an 8-mm trephine (Punch Biopsy). The injured tissue was then excised with curved sharp scissors down to the skull (head) and included the panniculus carnosus on the back. The excised skin served as internal-control, and was immediately divided for transcriptomics and backup. Specimens designated for transcriptomics analysis and those serving as backup were snap-frozen in liquid nitrogen and stored at −80 °C. A semi-occlusive dressing (Tegaderm^{TM}, 3 M, St. Paul, MN, USA) was applied to the wound after skin excision to avoid desiccation. The dressing was attached to the skin at the wound margins with 5–0 Prolene. The transparent nature of the material allowed for visual examination and photography of the wound. The wounds were then left to heal by secondary intention, i.e., the wound edges were not closed with sutures. On Day 4, 7, and Day 21, mice of the corresponding groups (4–5 mice per group) were anesthetized and skin samples of the wound area were excised and processed as described above. Mice were sacrificed using a lethal dose of the above anesthetics and skin samples processed on the designated end-point day (Day 4, 7 or 21).

Measurement of wound area

The anesthetized mice were placed in a well-lit room, on a table with a white background. At least five digital photographs (Olympus SLR, Shinjuku, Tokyo, Japan) of the wound area were taken for each mouse at indicated time points. Two types of pictures were taken: (i) Close-up pictures for thorough visual evaluation of the wound/scar; (ii) pictures taken from a distance of 25 cm while a ruler was aligned next to the wound (for wound area measurement). The morphometric analysis of wound closure was performed in a double-blinded fashion. Quantification of the wound area was carried out using the open source NIH ImageJ v1.43 software. Scale was set separately for each image, according to the ruler, using the ImageJ straight tool (ImageJ, NIH, http://www.nih.gov/).

Gene expression array

Approximately 30 mg of tissue was lysed in RLT-buffer (Qiagenp/n 80204) in a FastPrep FP120 instrument (Qbiogenep/n 6001-120) and extracted using Qiagen All-Prep DNA/RNA extraction kit (Qiagenp/n 80204), according to the manufacturer's instructions. RNA quality was assessed by the Agilent 2100 Bioanalyser (p/n G2938C). RNA samples were labeled using the Agilent low RNA input fluorescent linear amplification kit (p/n 5184–3523), according to the manufacturer's instructions. Briefly, 200 ng of total RNA was reverse transcribed. Amplification and labeling were performed by T7-polymerase in vitro transcription to produce Cy3-labeled cRNA. The dye incorporation rate was assessed with a Nanodrop ND-1000 spectrophotometer and was consistently >9 pmolCy3/μgRNA. Single color hybridizations were carried out using the Agilent Gene Expression Hybridization kit (p/n 5188–5242), according to the manufacturer's instructions. 1650 ng of cRNA was subjected to fragmentation (30 min at 60 °C) and then hybridized to 4 × 44 K Human Whole-Genome 60-mer oligo-chips (G4112F, Agilent Technologies) in a rotary oven (10 rpm, 65 °C, 17 h). Slides were disassembled, washed in solutions I and II, according to the manufacturer's instructions, and dried using Acetonitril. Scanning was done by an Agilent microarray scanner (p/n G2565BA) followed by Agilent Feature Extraction Software. Raw data was background corrected, quantile normalized and $\log_2$-transformed using R/Bioconductor (http://www.bioconductor.org (Gentleman et al. 2004)). Differential expression was calculated using a linear model of the limma software package (Smyth 2004).

Pathway enrichment

The k-means clustering algorithm was employed to group concordantly expressed genes (utilizing a linear model on the basis of F-statistic; $p < E-4$). Each of these gene clusters was subsequently subjected to Gene Set Enrichment Analysis against the KEGG pathway (Kanehisa et al. 2016; http://www.genome.jp/kegg/), the Reactome pathways (Fabregat et al. 2016; http://reactomerelease.oicr.on.ca/) and the Gene Ontology (GO) (Ashburner et al. 2000; http://geneontology.org/) databases. For gene classification and sorting, we used the PANTHER classification system (Mi et al. 2005; http://pantherdb.org/), the DAVID Bioinformatics Resources 6.7 (Huang et al. 2009), and the EnrichR toolset (Chen et al. 2013; http://amp.phafearm.mssm.edu/Enrichr/).

Results

Age-related differences in gene expression of intact skin

Using intact skin as an internal control during wound healing allowed us to gain insight into the gene expression changes in the middle-aged skin. Both the head and back areas showed differential gene expression in the middle-age group versus the young (Fig. 1). However, the significance of the gene expression changes was much higher in the head than in the back (Fig. 1a). Accordingly, the total number of differentially expressed genes ($p < 0.001$ after Benjamini correction) was much higher in the head than in the back (Fig. 1b). Nevertheless, a high portion (168 of 385) of the genes differentially expressed in the back, were also differentially expressed in the head. Moreover, the vast majority of these common genes (95 %) display the same direction of change (i.e. up- or down-regulated). Of note, the common genes were remarkably enriched in DNA metabolism and repair KEGG pathways (Fig. 1c). Using the Reactome pathway database brought about essentially similar results and, in addition, revealed a significant enrichment in the Cell Cycle pathway (Online Resource 1). These results

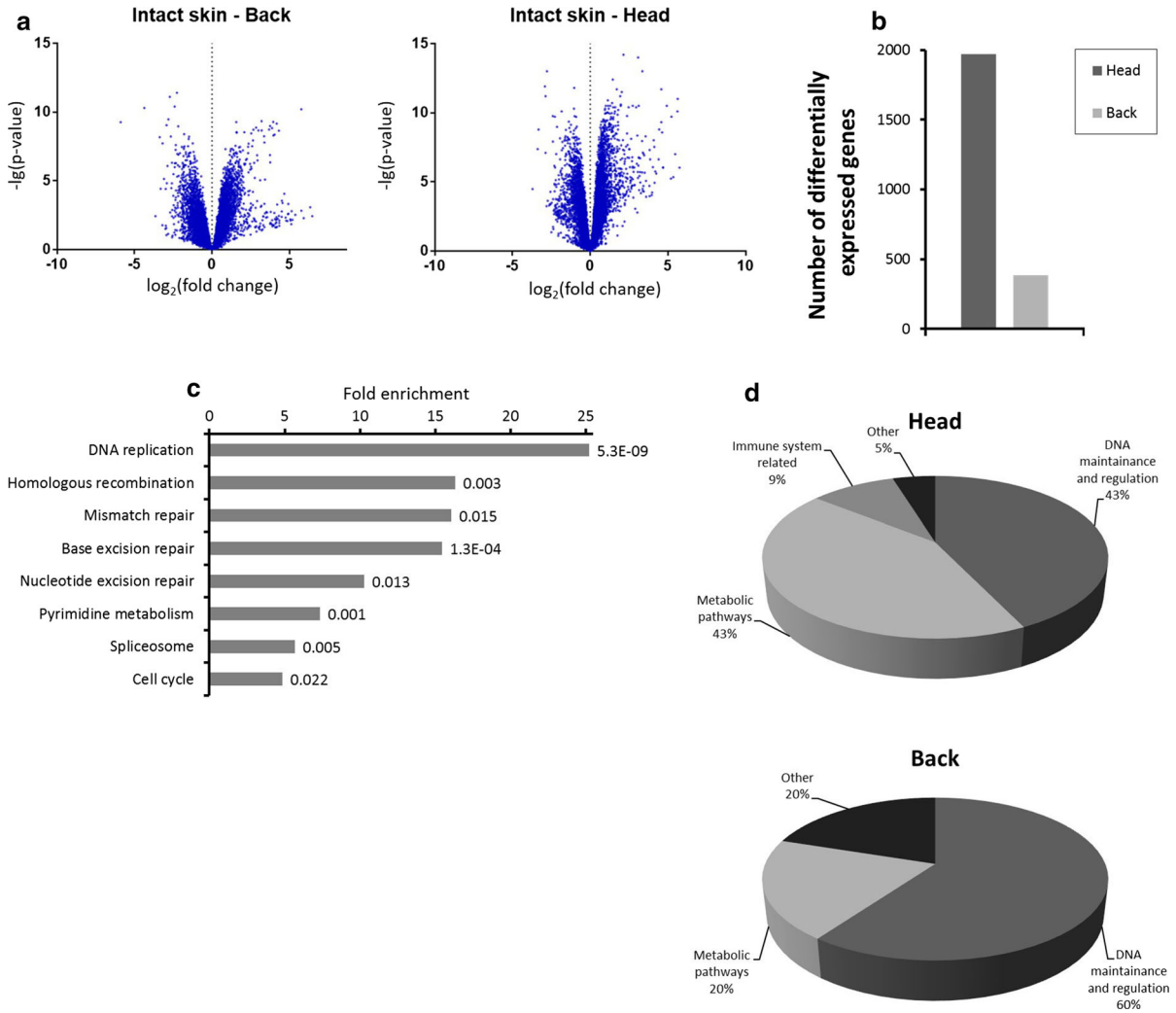

Fig. 1 Age-related differences in gene expression in intact skin of C57Bl/6 male mice. **a** Volcano plots (effect size) of middle-aged versus young mice. Effect size was plotted as $\log_2$ of fold change versus significance (B-value, i.e. log odds ratio of a gene being differentially expressed). Each *dot* represents an individual gene. **b** The number of genes differentially expressed in middle-aged versus young animals at Day 0 (intact skin) under stringent criteria ($p < 0.001$ after Benjamini correction). **c** List of enriched KEGG pathways among the 168 genes that are differentially expressed in both head and back at a high confidence ($p < 0.001$ after Benjamini correction). KEGG pathways are presented by order of fold enrichment. The p value after Benjamini correction is shown for each pathway next to the bar. **d** Distribution of enriched KEGG pathways. *Note* essentially the same results were obtained using the Reactome and Gene Ontology databases (For a full list of enriched categories see Online Resource 2)

were further supported by the Gene Ontology (GO) enrichment analysis (Online Resource 1). Interestingly, these common genes were also found to be highly interconnected (PPI enrichment p = 2.8E−13), and the enriched GO biological process categories form a continuous network via shared genes (Online Resource 1).

Analysis of the head and back separately (Fig. 1d; Online Resource 2) showed that a major difference between the aged head and back skin was the enrichment in immune response-related pathways. The majority of these genes encode for cytokines/chemokines or their receptors resulting in enrichment in Cytokine-cytokine receptor interactions,

Chemokine signaling pathways and inflammatory processes (Online Resources 3 and 4). Notably, many of the above mentioned immune-related genes are important for neutrophil activity which is known to be responsible for the initial inflammatory stage of WH (Guo and DiPietro 2010).

Gene expression during wound healing

As expected (for example, see Grose and Werner 2003; Yanai et al. 2015), for all groups examined, we consistently observed an overexpression of genes related to the extracellular matrix (ECM) including ECM organization, ECM-receptor interaction, collagen metabolism, and focal adhesion (Online Resource 5). Accordingly, the combined score which reflects both the fold enrichment and the statistical significance, was the highest for these categories in all groups and time points (Online Resource 5). Yet, along with common changes in gene expression, age had a marked impact on gene expression patterns during the course of skin WH. The age-related changes were especially noted for the head model and to a lesser degree for the back model. Throughout all stages of WH, middle-aged mice displayed a marked increase in metabolic pathways and starting on Day 7, also in growth-promoting pathways (Fig. 2, Online Resource 4). These included metabolic pathways, which were not enriched in the young (such as pyruvate metabolism, Fatty acid, lipid and lipoprotein metabolism, and glycolysis/gluconeogenesis) and growth-promoting pathways (such as Insulin signaling) (Online Resource 5).

Other interesting findings were related to the immune-inflammatory component of WH. In particular, the Fc gamma R-mediated phagocytosis pathway in the young was enriched only in the initial stage of WH (Day 4) but in the middle-aged mice, up to Day 7, indicating a prolonged inflammatory phase of WH (Online Resource 5). This is in line with the previous observation that prolonged wound healing in advanced age is associated with a reduced macrophage phagocytic capacity (Swift et al. 2001). Also, at Day 7 after wounding, the middle-aged animals exhibited a significant enrichment in the Toll-like receptor and the Adipocytokine signaling pathways (Online Resource 5). Both of these pathways involve TNFα, which is known to elongate the inflammatory phase in impaired WH (Guo and DiPietro 2010). Indeed, we found that 25 genes associated with TNFα induction (e.g. Tnfaip2, Tnfsf8, Tnfaip8l1, etc.) were differentially expressed 7 days after wounding in the middle-aged mice but not in the young. Among the topmost enriched categories were also leukocyte migration, cell chemotaxis, and regulation of inflammatory response (Online Resource 5). All of the above coincides well with a slower wound closure in the middle-aged head model, which was most obvious at Day 7, when the wound in the head of young mice closed to 28 ± 5 % of the initial size, but in the middle-aged mice it was twice as large (50 ± 5 %; $p < 0.05$; see Online Resource 6).

On Day 21 (i.e. approximately 10 days after full closure of the wounds; Online Resource 6), gene expression changes were much more pronounced in the head than in the back (Fig. 3). As a result, in the

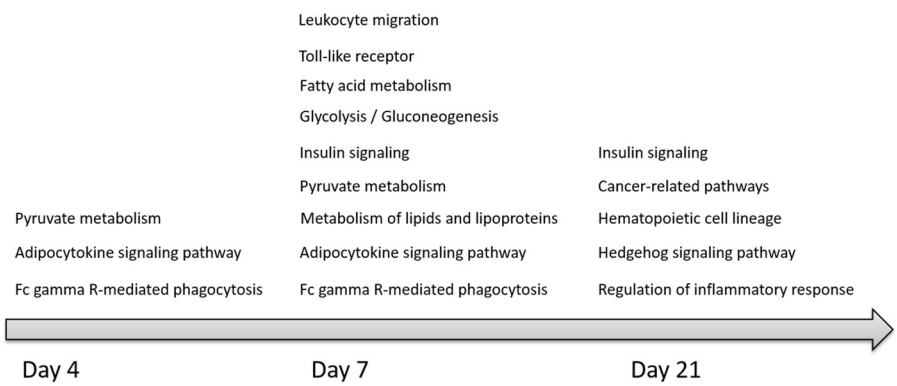

Fig. 2 Pathways enriched during wound healing in middle-aged but not in young mice. Presented are enriched categories among differentially expressed genes at indicated time points versus Day 0 ($p < 0.05$ after Benjamini correction with at least two-fold change in expression). For a full list of the enriched pathways, see Online Resource 5

head model, many pathways were still enriched by Day 21 (Online Resource 5). In particular, the middle-aged mice displayed a persistent enrichment in various metabolic and growth-promoting pathways.

Discussion

Mice are widely used in the study of cutaneous wound healing and in aging research in particular (Kim et al. 2015; Reid et al. 2004; Yanai et al. 2016). There are several in vivo murine skin wound healing models used in experimental research, each with its own merits (Reid et al. 2004; Wang et al. 2013). The most popular is the back excision model, which is readily applied for aging research, where the rate of wound closure is often used as an indicator of "biological age" (Yanai et al. 2011). Yet, one of the drawbacks of the back model is that wound closure mainly relies on wound contraction and to a lesser degree on the formation of new tissue. This is mostly due to a subcutaneous thin muscle layer, the *panniculus carnosus*, which is present in mice but not in humans (Greenwood 2010; Wong et al. 2011). Therefore, in this study, we also applied the head punch model which, as a result of the natural splinting effect of the skull, heavily relies on re-epithelialization from the wound edges (Reid et al. 2004; Gurtner et al. 2008).

Specifically, we focused on gene expression changes in the course of skin WH in late middle-aged C57BL/6 mice, which are roughly comparable in age to 60-year-old humans (Freitas et al. 2011). The reasoning behind our choice of this age group is that changes that manifest later in life, most probably have their origin in the middle age. The current consensus on cutaneous WH is that while many specific processes alter with age, "healing in the elderly is delayed but the final result is qualitatively similar to that in young subjects" (Gosain and DiPietro 2004). In line with this notion are our results showing that middle-aged mice did not exhibit any visible qualitative changes (such as hypertrophic scars, etc.), but did exhibit a delayed WH response. The latter was however observed only in the head wounds.

Since the head model relies more on cellular WH processes rather than mechanical ones, it is reasonable to expect more pronounced gene expression changes in the head than in the back. Apart from common gene expression changes in the head and back models (such as those relating to cytokine–cytokine interactions and hematopoietic cell lineage; see Online Resource 4), the head consistently presented a significantly higher number of differentially expressed genes from diverse pathways, during all stages of WH. The age-related differences were mostly observed for metabolic and growth-promoting pathways, and immuno-inflammatory pathways such as the Fc gamma R-mediated phagocytosis, the adipocytokine signaling, leukocyte migration, cell chemotaxis, and the Toll-like receptor pathway. The latter could, at least in part, be responsible for the observed delay in wound closure due to a prolonged inflammatory phase in the middle-age mice.

Another interesting and unexpected observation was that in our mice, only in the head model, gene expression changes persisted up to the resolution

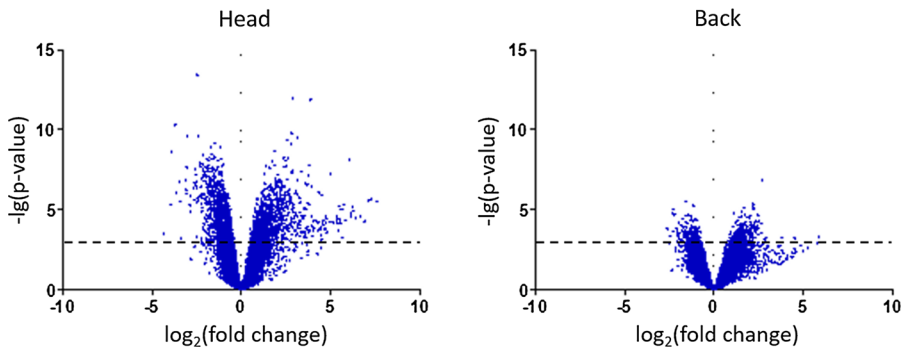

Fig. 3 Gene expression after full wound closure in middle-aged mice. Volcano plots (effect size) of middle-aged mice at Day 21 versus Day 0. Effect size was plotted as $\log_2$ of fold change versus significance (B-value, i.e. log odds ratio of a gene being differentially expressed). Each *dot* represents an individual gene. *Dotted line* represents p-value after Benjamini correction equals to 0.001

phase of wound healing (Day 21). At this time point, middle-aged mice displayed a persistent activation of genes involved in cell proliferation (such as PPAR signaling pathway and various cancer-related pathways) and the generation of new tissue (such as Hedgehog signaling pathway, Hematopoietic cell lineage, and epithelium development) (see Online Resource 5), pointing to an ongoing tissue remodeling. In contrast to the head model, gene expression in the back skin after wound closure returned to levels close to the intact skin.

The age-related changes in gene expression in the intact skin could be considered a predisposing factor for the observed changes in WH between the age groups. With regard to this notion, the impact of middle age was more significant for the skin of the head than of the back. Interestingly, the observed number of differentially expressed genes in the mouse head skin is comparable to that found in elderly humans (1967 and 1672, respectively; Glass et al. 2013). It is worth mentioning that the gene expression signature in the head skin of middle-aged mice has a high similarity with that of DNA demethylation in fibroblasts (MARQ analysis; data not shown). This could, at least in part, explain why we observed a higher number of upregulated genes in the head of middle-aged versus young mice (see Fig. 1).

Another point for future investigation arised from a recent study by Ansell et al. (2011) who showed that the mouse hair cycle in the intact skin may affect wound healing. While in this study we did not quantify the hair cycle phase, indirect evidence points to a possibility of such an effect in the middle-aged mice. To get some insight into this aspect, we searched whether the age-related transcriptional changes in the head skin have any similarity with those reported for the hair cycle. Using the MARQ analysis tool (Vazquez et al. 2010; http://marq.dacya.ucm.es/; data not shown), we found some similarity between the transcriptional age-related changes in intact skin and those relating to hair follicle development. This similarity was even stronger when comparing the gene expression patterns at Day 7 after wounding (when the difference in healing rate between the age groups was most significant and the prolonged inflammation in wounded skin of the middle-aged mice was evident). While we cannot state the functional significance of the hair cycle in age-related differences in skin WH, the data points towards such a possibility.

In this study, we used male mice to avoid the possible effects associated with variations in the estrous cycle. Yet, the gender factor is an interesting aspect of wound healing and age. Indeed, endogenous sex hormones profoundly influence both the rate of aging and the response to cutaneous injury (Gilliver et al. 2008), so that female mice generally live longer and heal better than males. Likewise, castration of male mice extends their life span and improves wound repair. In our previous study on female FVB/N mice (Yanai et al. 2015), we found clear differences in the rate of head skin wound closure and gene expression between young and old females. It would be interesting to examine if our observations are relevant for middle-aged C57BL/6 female mice as well.

In summary, the middle age has a significant impact on gene expression both in intact skin and during all phases of skin WH. Yet, these age-related changes were, to a great extent, area-specific, being much more pronounced for the head skin than for the back. Although our results are based on transcriptional changes of high significance, further validation of our observations by qPCR as well demonstration of the expression changes at the protein level, at least for specific genes, is warranted. Nevertheless, it seems that the head punch model is more sensitive to the effect of age, and we suggest that this model should be more widely applied in aging research of wound healing.

Acknowledgments This study was funded by the European Union FP7 Health Research Grant number HEALTH-F4-2008-202047, and by the Israel Ministry of Science and Technology. This work was also supported by the Fund in Memory of Dr. Amir Abramovich.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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