VILNIUS UNIVERSITY
STATE RESEARCH INSTITUTE
CENTRE FOR INNOVATIVE MEDICINE

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INVESTIGATION OF THE INFLUENCE OF BONE MARROW STEM CELLS ON SKIN REGENERATION IN BALB/c MOUSE MODEL *IN VIVO*

Summary of doctoral thesis
Biomedical sciences, biology (01B)
Immunology, serology, transplantation (B500)

Vilnius, 2014

This study was carried out in 2009-2013 at the State Research Institute Centre for Innovative Medicine.

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Defense of the doctoral dissertation will take place at the open meeting held by the Research Board for Biology at 11 a.m. on 3 July 2014 in the hall of State Research Institute Centre for Innovative Medicine. Address: Molėtų pl. 29, Vilnius, Lithuania.

The summary of dissertation was sent on 3 June 2014.

The dissertation is available at the Library of Research Institute Centre for Innovative Medicine and Vilnius University Library.

VILNIAUS UNIVERSITETAS
VALSTYBINIS MOKSLINIŲ TYRIMŲ INSTITUTAS
INOVATYVIOS MEDICINOS CENTRAS

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KAULŲ ČIULPŲ KAMIENINIŲ LĄSTELIŲ ĮTAKOS ODOS REGENERACIJAI TYRIMAS BALB/c LINIJOS PELIŲ MODELYJE *IN VIVO*

Daktaro disertacija
Biomedicinos mokslai, biologija (01B)
Imunologija, serologija, transplantacija (B500)

Vilnius, 2014

Disertacija rengta 2009-2013 m. VMTI Inovatyvios medicinos centre.

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Disertacija bus ginama viešame Biologijos krypties tarybos posėdyje 2014 m. liepos 3 d. 11 val. VMTI Inovatyvios medicinos centro posėdžių salėje. Adresas: Molėtų pl. 29, Vilnius, Lietuva.

Disertacijos santrauka išsiųsta 2014 m. birželio 3 d.

Su disertacija galima susipažinti VMTI Inovatyvios medicinos centro ir Vilniaus universiteto bibliotekose.

Introduction

The events of each wound healing phase must happen in a precise and controlled manner. Interrupted, aberrant or prolonged reactions in the healing process can lead to a non-healing chronic wound (Guo and DiPietro, 2010). Current strategies used in clinical practice for the treatment of non-healing wounds are skin grafting and application of skin substitutes (Chen et al., 2009). In the first case, autografts remain the best choice. However, in extensive skin injuries, the availability of donor sites is extremely limited. Main disadvantage of allografts and xenografts is inevitable immunologic rejection of the graft. Prevention of transplant rejection requires immunosuppressive therapy, which has a strong negative side effect (Zhou et al., 2013). Bioengineered skin substitutes represent an alternative to skin grafts. Skin substitute biomaterials can be either natural or synthetic, must evoke minimal inflammatory reactions and should be biodegradable (Polak and Bishop, 2006). The majority of current commercially available skin substitutes serve as the primary barrier against infection and dehydration. However, they are not capable of promoting tissue regeneration, therefore wound healing results in scar formation and restoration of partially functional skin. Stem cell therapy can improve the quality of wound healing and re-establish the function of native skin tissue (Muscari et al., 2013).

Success of regenerative medicine depends on efficient differentiation of stem cells into specific lineages (Baraniak and McDevitt, 2010). Embryonic stem cells (ESCs) are able to create all types of mature cells, but their application in medicine is limited due to the ethical issues and the lack of knowledge in directing their differentiation ability (Vazin and Freed, 2010). Adult stem cells are promising alternative to ESCs. Adult bone marrow contains two distinct types of stem cells: hematopoietic stem cells (HSCs), responsible for production of all blood cell types, and mesenchymal stem cells (MSCs), which are able to differentiate into bone, cartilage and adipose tissue. Beside multipotent stem cells, bone marrow contains also a heterogeneous population of progenitor cells with regenerative potential (Wu et al., 2010).

Several clinical studies showed a significant decrease in wound size after MSCs application in the treatment of nonhealing ulcers (Vojtassák et al., 2006; Falanga et al., 2007; Ravari et al., 2011). Endothelial progenitor cells (EPCs) have been intensively investigated during past years because of their role in neovascularization. The formation of new vessels in wound healing is crucial for the supply of nutrients (Watt et al., 2010).

Preclinical studies have demonstrated the ability of HSCs to participate in cutaneous wound healing. *In vivo* studies have shown that during the early inflammatory phase of wound healing, there is a significant increase of bone marrow-derived cells originated from HSCs population (Fathke et al., 2004). Skin wound healing involves the interactions between different cell types, therefore acceleration of regeneration process requires the population of multifunctional cells (Sorrell and Caplan, 2010). Although the progress in stem cell research has been much improved, there are still many problems that need to be resolved before these cells can be widely used clinically. Data from preliminary studies are insufficient to show long-term effect of transplanted cells. It is not clear what factors influence the engraftment of transplanted cells in the wound area, accelerate their adaptation to the foreign microenvironment and ensure long-term functions. Further intensive studies are required to solve these problems (Charruyer and Ghadially, 2009). The choice of the accessible source to obtain a sufficient cell amount and the use of suitable biomaterials to improve the cell delivery efficiency are the main tasks for safe, effective and reliable application of stem cell therapy (Huang and Burd, 2012).

In this study, we investigated the influence of bone marrow-derived lineage negative (Lin^-) cells on skin tissue regeneration after local transplantation with different biomaterials *in vivo*. Results of the present study are important for the development of new techniques to treat cutaneous wounds.

The aim of dissertation work

To investigate the influence of bone marrow Lin^- cells on skin regeneration in BALB/c mice full-thickness skin wound model *in vivo*.

The objectives of this work were as followed:

1. To purify and identify bone marrow Lin^- cell population of the BALB/c mice.
2. To establish the influence of skin extracellular components on Lin^- cell proliferation and migration *in vitro*.
3. To evaluate the integration of Lin^- cells into the wound tissue after local transplantation with different biomaterials and their effect on skin wound healing *in vivo*.

4. To determine the influence of Lin^- cell on gene expression of cytokines during wound healing *in vivo*.

Scientific novelty

The use of bone marrow-derived stem/progenitor cells in the treatment of nonhealing wounds is promising because of their high potential to form differentiated cells. A significant progress has been made in understanding the influence of distinct stem cell populations on skin regeneration. However, the survival of transplanted cells in the wound site, their long-term activity and effective restoration of functional tissue are the questions still to be discussed. In the present study, regenerative properties of mouse bone marrow-derived Lin^- cell population combined with various components of skin tissue extracellular matrix were compared.

The phenotypic analysis of Lin^- cells was carried out. The influence of type I collagen and basement membrane components on cell proliferation and migration *in vitro* was investigated. For the first time the effect of Lin^- cells on skin tissue regeneration after local transplantation with different biomaterials was evaluated in the BALB/c mouse full-thickness wound model *in vivo*. It was found that biomaterials used for cell delivery ensure integration and survival of transplanted cells in the wound area, affect their role in inflammation, re-epithelization and formation of skin appendages. In the present study, the gene expression of cytokines, important for wound healing, after local Lin^- cells transplantation was compared.

Our results enlarge the knowledge required to develop methods for the improvement of skin tissue regeneration.

The defensive statements

1. Bone marrow Lin^- population contains undifferentiated cells with regenerative properties.
2. Skin extracellular matrix components influence proliferation and migration of Lin^- cells *in vitro*.
3. The influence of Lin^- cells on skin regeneration *in vivo* depends on the biomaterial used for local transplantation.

Dissertation contents. The dissertation is written in Lithuanian and contains the following parts: Introduction, Literature review, Methods, Results and Discussion, Conclusions, List of publications, List of conference reports, List of references (209 positions), Tables (5), Figures (21). Total pages 119.

Materials and methods

Laboratory animals. BALB/c mice (20-25 g) were used. Animals were housed at 22±2°C under a 12-h light-dark cycle and with free access to food and water. All procedures were approved by Lithuanian Ethics Committee on the use of laboratory animals under the State Veterinary service (No. 0208/ 2011).

Isolation of mouse bone marrow cells. Bone marrow cells were isolated from femur and tibiae of BALB/c mice 8 weeks of age as described by Juopperi et al., (2007) with some modifications. Mice were sacrificed by cervical dislocation. The extracted bones were placed on a Petri dish containing phosphate buffered saline (PBS). Bone marrow was obtained by flushing with sterile PBS through one of the epiphyses, using a syringe needle (27-gauge). The bone marrow cells were collected in sterile PBS and washed three times by centrifugation for 6 min at 300 g.

Preparation of Lin^- cell population. Bone marrow cells were purified using magnetic cell sorting (MACS) techniques with the BD IMag™ mouse hematopoietic progenitor enrichment set (BD Biosciences, USA) applied as recommended by the manufacturer. Total bone marrow cell suspension (2×10^7 cells/ml) was prepared in sterile PBS with 3% FCS. Cells were incubated on ice for 15 min with anti-mouse CD16/CD32 to block Fc receptors, and then incubated on ice for 15 min with a mix of biotin-conjugated monoclonal antibodies: anti-mouse CD3e, clone 145-2C1; anti-mouse CD11b, clone M1/70; anti-mouse CD45R/B220, clone RA3-6B2; anti-mouse Ly-6G and Ly-6C (Gr-1), clone RB6-8C5; anti-mouse TER-119, clone TER-119. After washing, cells were resuspended in IMag™ Streptavidin Particles Plus and incubated at 6°C for 30 min. Labeled cells were transferred to a test tube and placed on the BD IMagnet™ for 8 min. With the tube on the BD IMagnet™, supernatant (depleted Lin^- fraction) was carefully aspirated and placed in a new sterile tube. Positive fraction was resuspended in BD IMag™ buffer and procedure on the magnet was repeated. Final depleted Lin^- fraction was washed in PBS by centrifugation for 6 min at 300 g and used for further experiments.

Phenotypic characterization of Lin^- cells. Purified Lin^- cells (5×10^5) were incubated for Fc-receptor blockage by CD16/CD32 antibody (BD Biosciences, USA) at 4°C for 5 min and then stained with phycoerytrin (PE)-, allophycocyanin (APC)- or fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against cell surface markers. Following antibodies were used: hamster anti-mouse CD29-APC (Miltenyi Biotec, USA), rat anti-mouse Sca-1-FITC (Molecular Probes, USA), rat anti-mouse CD117-APC (Molecular Probes, USA), rat anti-mouse CD133-FITC (Santa Cruz Biotechnology, USA) and rat anti-mouse CD90.1-PE (BD Pharmingen, USA). Isotype control antibodies hamster IgG-APC, rat IgG2a-FITC, rat IgG2b-APC and rat IgG1-FITC/PE were used. Cells with antibodies were incubated at 4°C for 30 min in the dark, washed in PBS by centrifugation for 6 min at 300 g and resuspended in 500 µl PBS. Analysis was performed with FACSCalibur™ flow cytometer (Becton Dickinson, USA).

Lin$^-$ cell proliferation assay *in vitro*. Cell proliferation was assayed with the Cell Counting Kit-8 (CCK-8) according to the manufacturer's protocol (Dojindos Laboratories, Japan). In culture method 85 µl of Geltrex (Gibco, USA) or 35 µl of 4 mg/ml rat tail collagen I (Gibco, USA) was added per well of 96-well plate and incubated at 37°C for 30 min to promote gelling of matrix. Purified Lin$^-$ cells were suspended in growth medium containing 2% of Geltrex or 2% of 5 mg/ml collagen I solution, and 10^4 cells/well were plated. For the control study, Lin$^-$ cells in growth medium were transferred directly to a 96-well microplates without type I collagen or Geltrex and were maintained under same conditions as the other groups. All cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. An appropriate amount of WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] reagent was added at the time point of 24, 48, 72 and 96 hours after seeding and incubated at 37°C for 4 hours. Tetrazolium salt is reduced by dehydrogenases in cells to give an orange colored formazan which is soluble in the culture medium. The amount of formazan dye is directly proportional to the number of living cells. The optical density (OD) values were read at 450 nm by a microplate reader.

Lin$^-$ cell migration assay *in vitro*. Scratch assay *in vitro* was performed as described by Liang et al., (2007) with some modifications. For surface coating, Geltrex was diluted to 0.5 mg/ml concentration in ice-cold serum-free medium, and collagen type I was diluted to 50 µg/ml concentration in 0.02 M acetic acid. The sufficient amount of prepared solution was added into each well of a 24-well plate to cover the entire growth area. Coated plate was incubated at 37°C for 60 minutes. Before cell seeding, collagen-coated wells were rinsed three times with media to remove the acid. Purified Lin$^-$ cells were plated at a density 5×10^5 cells/well in 1 ml of growth medium. For the control study, Lin$^-$ cells in growth medium were transferred directly to a 24-well microplates without type I collagen or Geltrex. All cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. Cells were grown to confluence. A single stripe was scraped on the cell-coated surface with a 200 µl disposable plastic pipette tip, and detached cells were removed by washing with medium. Wound closure was monitored by phase-contrast microscopy at the indicated time points. The images were captured with a Nikon DS-2MBW camera connected to a Nikon ECLIPSE TE2000-U microscope. Wound closure percent was determined by the following formula:

Wound closure (%) = $[(\text{Wound area}_{0h} - \text{Wound area}_{xh})/\text{Wound area}_{0h}] \times 100$.

Lin$^-$ cell labeling with fluorescent dye. Purified cells were labeled with the PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich, USA) applied as recommended by the manufacturer. Cells (2×10^7) were suspended in 1 ml of commercial Diluent C, and stained by mixing with an appropriate amount of freshly prepared 4 µM PKH26 working solution. Staining was stopped after 5 min by adding 2 ml of FCS. Cells were centrifuged for 10 min at 400 g, supernatant was removed and cell pellet was washed 2 times by centrifugation with growth medium. After final washing, cells were suspended in appropriate medium and used for transplantation *in vivo*.

Wound model *in vivo*. Full-thickness skin wounds were created as described by Wong et al., (2011) with some modifications. Mice were individually anesthetized using an subcutaneous injection of 0.5% bupivacaini hydrochloricum (15µl/mouse). Skin was shaved, cleaned, and disinfected with 70% alcohol. Excisional 6-mm full-thickness skin wound was created on the right side of the midline using sterile biopsy punch. The wound was left uncovered.

Lin^- cell transplantation *in vivo* and biopsy processing. The animals with excisional full-thickness skin wounds were divided into six groups, 8 per group. Groups 1, 2 and 3 received 1×10^6 Lin^- cell in PBS, type I collagen gel (1.5 mg/ml) or Geltrex gel (10 mg/ml), respectively. Control groups 4, 5 and 6 received proper solution or gel without cells. Lin^- cells suspensions or solutions without cells were injected intradermally around the wound at four injection sites. Mice were sacrificed at different time points, and skin samples including the wound and 2 mm of the surrounding skin were harvested using 8-mm biopsy punch. One-half of each sample was snap-frozen in liquid nitrogen immediately and stored at -76°C until cryosection. The other half was fixed in 10% neutral-buffered formalin for paraffin section. Histological sections were prepared in cooperation with the National Centre of Pathology.

Wound analysis. Digital photographs were taken on the day of surgery and every day thereafter. Time to closure was defined as the time at which the wound bed was completely filled in with new tissue. Wound size was calculated using the formula $(D_n/D_o)\times100\%$, where D_o is the dimension of the full-thickness skin wound area (6 mm diameter) on day 1 and D_n is the dimension of the wound area on indicated day. For fluorescent mocroscopy analysis, 5 μm thick sections were stained with 1 μg/ml DAPI (Molecular Probes, USA). The sections were observed under a fluorescent microscope Nikon Eclipse TE-2000-U (Nikon Corporation, Japan). PKH26-labeled cells appeared red and nuclei stained by DAPI were blue. Serial 5 μm paraffin sections were stained with hematoxylin/eosin or Masson‘s Trichrome, and observed under a light microscope Nikon Eclipse 50i (Nikon Corporation, Japan). Cell infiltration, re-epithelization, formation of granulation tissue and remodeling of extracellular collagen were evaluated.

RNA extraction. Total RNA was isolated from wounded skin samples. Tissue (25 mg) was lysed in lysis solution (Roche Molecular Systems, USA). For RNA isolation, 200 μl of lysate was mixed with 40 μl of protease and 80 μl of MGP (both from Roche Molecular Systems, USA), and incubated at 56°C for 10 min. The sample was cooled, centrifuged for 30 sec at 16150 g, and supernatant was removed using a magnet. MGP pellet was mixed with 500 μl of wash buffer AW1 (Qiagen, Germany), centrifuged for 30 sec at 16150 g, and supernatant was removed using a magnet. MGP pellet was mixed with 500 μl of wash buffer AW2 (Qiagen, Germany), centrifuged for 30 sec at 16150 g, and supernatant was removed using a magnet. MGP pellet was dried at RT for 30 min and 30 μl of elution mix (Fermentas, Lithuania) was added. The sample was mixed, transferred to a new tube and incubated at 37°C for 30 min. Reaction was terminated by incubating with 3 μl of 50 mM EDTA at 65°C for 12 min. The sample was cooled, mixed with 67 μl of TE buffer (Carl Roth GmbH, Germany) and 100 μl of 4 M LiCl solution, and incubated at -20°C for 30 min. The sample was centrifuged for 20 min at 16150 g, and supernatant was removed using a magnet. MGP pellet was mixed with 100 μl of 2 M LiCl solution centrifuged for 5 min at 16150 g, and supernatant was removed using a magnet. MGP pellet was mixed with 200 μl of ice cold 70% EtOH, centrifuged for 5 min at 16150 g, and supernatant was removed using a magnet. Step with EtOH was repeated. MGP pellet was dried at RT for 30 min and 20 μl of nuclease-free H_2O was added. After incubation at RT for 2 min, the sample was mixed and the eluate was transferred to a new tube using a magnet.

Reverse transcription (RT) reaction. First-strand cDNA was synthesized using High Capacity cDNA Reverse Transcription kit with RNAse Inhibitor (Applied Biosystems, USA) according to the manufacturer's instructions. RT reactions were performed in a total

volume of 20 µl containing 10 µl of isolated RNA, 2 µl of 10X RT buffer, 0,8 µl of 25X dNTP mix, 2 µl of 10X random primers, 1 µl of reverse transcriptase, 1 µl of RNAse inhibitor and 3,2 µl of dH_2O. After the initial step at 25°C for 10 min, RT reaction was performed at 37°C for 120 min. Reaction was terminated by heating at 85°C for 5 min. cDNA was cooled to 4°C temperature and stored at -20°C until further analysis.

Polymerase chain reaction (PCR). cDNA samples were amplified by PCR using AmpliTaq Gold® Fast PCR Master Mix, UP (2X) (Applied Biosystems, USA) according to the manufacturer's instructions. Amplification reactions were performed in a total volume of 15 µl containing 4 µl of template DNA, 0,3 µl of primers (Table 1), 3.2 µl of SYTO 9 dye and 7.5 µl of 2X PCR master mix. After initial denaturation at 95°C for 10 min, amplification consisting of denaturation at 95°C for 10 sec, annealing at 57-60°C (depending on primer) for 60 sec and extension at 68°C for 30 sec was performed for 40 cycles followed by final extension at 72°C for 10 sec. All real-time PCR reactions were done with thermal cycler Rotor-Gene Q (Qiagen, Germany). Results were demonstrated as mRNA expression in the wounds relative to non-wounded skin. The relative levels of gene expression were calculated by reference to the β-actin in each sample, using the cycle threshold (C_t) method. Amplified fragments were separated by electrophoresis in a 1.2% agarose gel and detected by GelRed fluorescent dye under UV transilluminator (Consort, Belgium).

Table 1. Primers for cDNA amplification by PCR.

	Primer	**Sequence**	**Product size, bp**	**Source**
β-actin	Forward	5'ACGGCCAGGTCATCACTATTG3'	103	Bing et al., 2006
	Reverse	5'CAAGAAGGAAGGCTGGAAAAGA3'		
TGF-β	Forward	5'CACCTGCAAGACCATCGACA3'	241	Szpaderska et al., 2003
	Reverse	5'CACGCGGGTGACCTCTTTAG3'		
VEGF	Forward	5'TGAACTTTCTGCTCTCTTGG3'	457	Mori et al., 2002
	Reverse	5'AACAAATGCTTTCTCCGCTC3'		
TNF-α	Forward	5'CAGCCTCTTCTCATTCCTGCTTGTG3'	511	Mori et al., 2002
	Reverse	5'CTGGAAGACTCCTCCCAGGTATAT3'		
IL-10	Forward	5'CTGCTCTTACTGACTGGCATGAG3'	534	Ohshima and Sato, 1998
	Reverse	5'GACTCAATACACACTGCAGGTGT3'		

Statistical analysis. All assays were repeated at least in three independent experiments. Results were expressed as mean ± SD. Differences between groups were tested by Student's t-test. Results were considered statistically significant when $p < 0.05$.

Results and Discussion

Phenotypic analysis of bone marrow-derived Lin^- cells.

Bone marrow-derived undifferentiated cells form a heterogeneous population. However, their common property is the absence of cell surface markers specific to differentiated cells. This population is generally defined as lineage negative (Lin^-) (Dawn and Bolli, 2005). In our study, Lin^- cell population was isolated from the total bone marrow cell count by negative selection using a magnetic-activated cell sorting (MACS). To eliminate differentiated cells, magnetic nano particles conjugated with antibodies

against lymphoid, myeloid and erythroid cell surface markers were used. The results obtained show that after purification of the total bone marrow cell population, 3.5±0.9% cells remained in the negative fraction. To test the purity of this cell population, the isolated Lin^- cell suspension was stained with monoclonal antibodies against the differentiated cell surface markers and further analyzed by flow cytometry. The results revealed that the presence of surface markers in the purified cell population was less than 2±0.8%. As there is no one unique surface marker that could define a specific stem cell or cell progenitor population, the necessity arises to combine several cell surface molecules in order to determine the phenotype of the primitive cells. The expression of a certain marker may also vary depending on the cell differentiation stage as well as it might be expressed by cells of the different origin (Herzog et al., 2003). To identify the undifferentiated cells in a Lin^- cell population, the following markers have been selected – CD117, Sca-1, CD90.1, CD29 and CD133. The expression of CD117 was found to be the highest in the purified cell population, up to 81.89±2.92% cells (Fig. 1A) expressed this marker. The expression of the remaining four markers in the Lin^- cell population was significantly lower. It was detected that Sca-1 molecule was expressed on 16.46±3.51% cells (Fig. 1B); CD90 – 9.48±2.93% (Fig. 1C); CD29 – 5.53±1.8% (Fig. 1D) and CD133 – 6.64±0.93% (Fig. 1E).

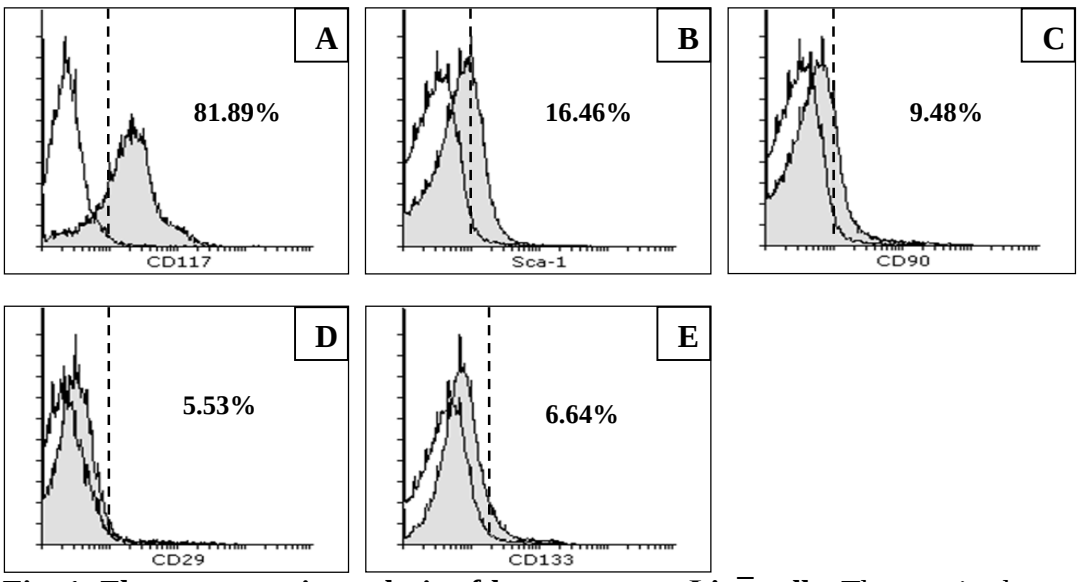

Fig. 1. Flow cytometric analysis of bone marrow Lin^- cells. The x-axis shows fluorescence intensity of fluorochrome-conjugated antibodies against cell surface markers; the y-axis indicates the cell number. White histograms show background staining with isotype control antibodies, and grey histograms show specific staining. Mean fluorescence intensities are indicated. Comparable results were obtained in five independent experiments.

To summarize the results from cell surface marker studies, we could conclude that the majority of the primitive cells from the Lin^- cell population belong to the hematopoietic cell line. The insignificant expression of mesenchymal origin undifferentiated cells show the necessity of their expansion *in vitro* in order to obtain the necessary amount for therapy. Data in literature reveal that HSCs and MSCs are not limited by their differentiation to specific mature cells. Cells at different differentiation stages, interacting in the bone marrow Lin^- cell population, supplement to the regenerative properties of each other, thus obtaining the preference to be used as a cell source for therapy.

Proliferation and migration of Lin^- cells in vitro.

A prolonged wound healing could be associated with the disorder of cell migration and proliferation processes that occur in the cells participating in the tissue restoration. These processes are directly influenced in the organism by the microenvironment (Watt and Fujiwara, 2011). To investigate the cell proliferation and migration *in vivo* is rather difficult because of the complex interactions that take part simultaneously (Amadeu et al., 2003). Therefore, the proliferative and migrational properties of mouse bone marrow Lin^- cell population were tested *in vitro*. The microenvironment was formed on the basis of the extracellular components of the skin tissue. Type I collagen and basement membrane components (laminin, type IV collagen, entactin and heparan sulfate proteoglycan mixture) have been used.

The results showed the increased proliferation of the Lin^- cell population during observation period in the microenvironment possessing extracellular matrix components (Fig. 2). In the experimental group, where the cells were cultivated in the type I collagen gel, the intensity of proliferation was from 1.2- to 1.6-fold higher compared with the control. Using the basement membrane component gel, a 1.3- to 2-fold increase of cell proliferation was registered. As compared with the initial point (24 h), increased proliferation in both experimental groups was detected after different cultivation periods. Proliferation activity in type I collagen gel and basement membrane matrix was up to 1.5-fold higher after 72 h and 96 h, respectively. Lin^- cell proliferation in the control group was detected only during the first 72 h (Fig. 2). Data by other investigators prove that different extracellular matrix proteins at the conditions *in vitro* could affect the

proliferation activity of different cell types, engaged in tissue restoration, such as fibroblasts (Bott et al., 2010), keratinocytes (Visser and Pollitt, 2010) and distinct stem cell populations (Penolazzi et al., 2012). In our study, the most active proliferation of the Lin^- cell population was determined in the basement membrane matrix. In this group, the marked increase (up to 1.5-fold) in proliferation compared with type I collagen group was established after 48 h and 96 h (Fig. 2). Thus, it was proved that optimal cell adaptation and proliferation in a foreign microenvironment, as at the conditions *in vivo*, was maintained by the complex of extracellular components.

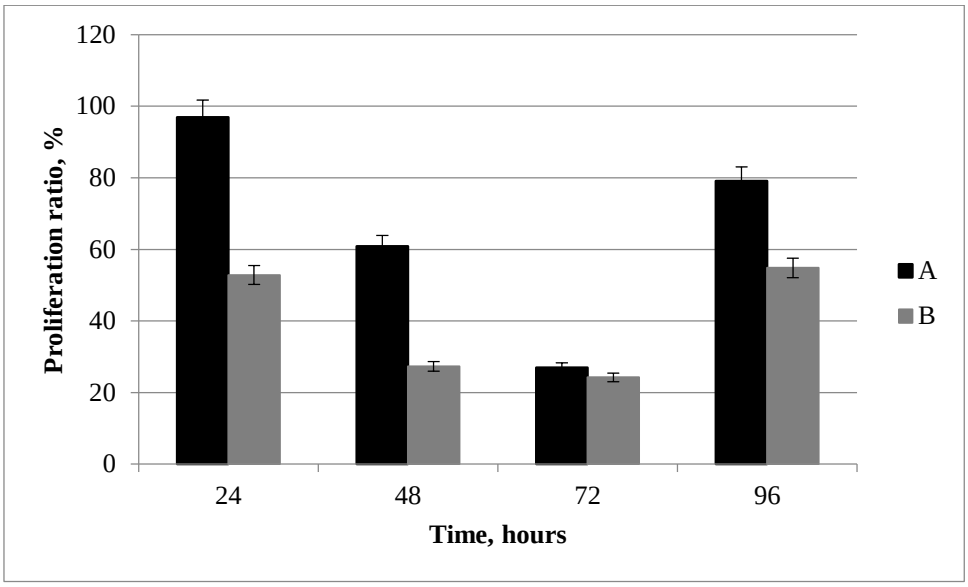

Fig. 2. Proliferation of Lin^- cells in different extracellular environments *in vitro*. (A) Cells in microenvironment, containing basement membrane components. (B) Cells in microenvironment, containing type I collagen. Data are presented as mean ± SD (n = 4); $p < 0.05$.

Cell migration is another important process that provides and ensures not only the developing of the tissue and its homeostasis, but also plays an important role in the successful wound healing (Thampatty and Wang, 2007). The kinetics of the Lin^- cell population *in vitro* was evaluated analyzing a cell monolayer, formed on type I collagen and basement membrane components in the scratch assay. The results are shown in Fig. 3. The data of other investigators on the different cell type migration *in vitro* are rather contradictory. Some studies indicate that the migration of keratinocytes is stimulated by a basement membrane component, type IV collagen. However, other components of this structure – laminines, just on the contrary, inhibit the process (O' Toole, 2001). Contradictory results were obtained in studies of fibroblast migration. Up to twice

increased migration of these cells was found in the microenvironment using the mixture of basement membrane components, but not with the type I collagen (Hakkinen et al., 2011). The experiments with the dental pulp stem cells revealed that migrating cells at *in vitro* conditions most rapidly covered the injured area on the surface of basement membrane components, but not that with separate proteins (Boleman et al., 2012). Our studies showed the stimulation of the Lin^- cell migration on the surface of basement membrane components during the first days of the experiment and its earliest completion in the microenvironment of type I collagen. After 24 hours, the wound closure was more expressed on the basement membrane matrix than on the surface monolayer of collagen type I. Skin injury in these groups decreased by 50.78% and 63.62%, respectively. As compared to the control group, the open wound area in the tested groups was decreased by 1.6 times. The smallest difference of the injured skin area among the groups tested was registered after 48 h. After 72 h, when the re-epithelization on collagen I type surface was complete, the size of the remaining injured surface on the basement membrane matrix was 24.89%. A complete wound closure by Lin^- migrating cells in this group was observed at 96 h post-wounding. In the control group, following 72 and 96 h, the re-epithelization process was not complete – 44.56% and 31.29%, respectively, of wounded surface area remained uncovered (Fig. 3).

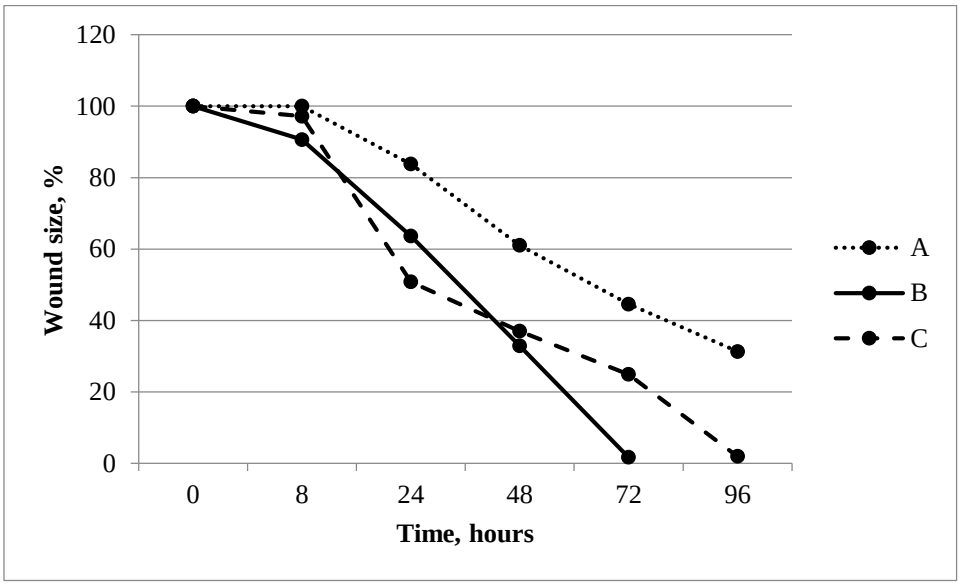

Fig. 3. **Migration of Lin^- cells in different extracellular environments *in vitro*.** (A) Control group. (B) Cells in microenvironment, containing type I collagen. (C) Cells in microenvironment, containing basement membrane components. Data are presented as mean ± SD (n = 4); $p < 0.05$.

To summarize the results obtained, we could state that regenerative properties of bone marrow-derived undifferentiated cells depend on the skin extracellular matrix components. Active Lin^- cell proliferation and enhanced migration in the microenvironment with type I collagen or basement membrane components show that for cell survival the interaction with the extracellular components is necessary. More expressed cell proliferation in the basement membrane matrix suggests that for cell adhesion and division a complex of components, recognized by cell surface structures, is required. Contrary to proliferation, Lin^- cell migration process requires the presence of type I collagen, which is the main component of the extracellular matrix.

The influence of Lin^- cells on skin tissue regeneration in vivo.

The effect of bone marrow Lin^- cell population, integrated into different biomaterials, for injured tissue regeneration was studied *in vivo* in BALB/c mice full-thickness skin wound model. Wound macroscopic analysis, qualitative test of the healing wound tissue and quantitative changes in the gene expression of cytokines at the skin injury site were tested.

The macroscopic analysis revealed the difference in wound closure in the control group and after Lin^- cell transplantation in PBS solution, in type I collagen gel or basement membrane matrix during the time course of the experiment (Fig. 4B, D and F).

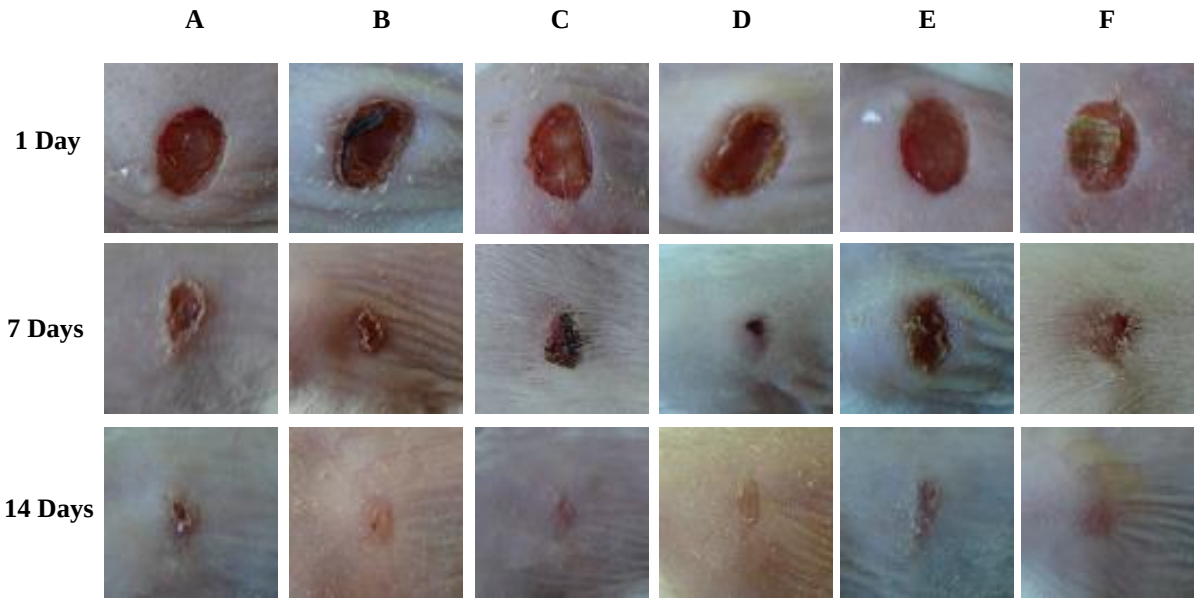

Fig. 4. Time course of wound closure. Lin^- cells (1×10^6) were injected into the wound with PBS (B), type I collagen gel (D), and basement membrane matrix (F). Control groups received PBS (A), type I collagen gel (C), and basement membrane matrix (E) without cells.

Among groups, where the injury was treated with Lin^- cells in different matrices, there were no significant differences in wound closure up to day 3. Statistically significant changes in these groups were registered only following day 4 after the cell transplantation and continuing till the end of the experiment. The biggest difference in wound closure among the groups was registered at day 10. In the group, where Lin^- cells were injected in combination with type I collagen gel, the open wound area was 4.2 times smaller than that when the cells were injected with PBS and 5.3 smaller as compared to the wounds treated with Lin^- cells in the basement membrane gel. The complete wound closure was detected the earliest at day 12 with the Lin^- cells in type I collagen gel. In the groups, treated with Lin^- cells suspended in PBS solution or basement membrane component gel, the wound area remained uncovered by 8.08±0.26% and 9.58±0.57%, respectively. The complete wound closure in these groups was registered at day 14 (Fig. 4). Some studies suggest that after treatment with different stem cell populations the impaired wound healing area is significantly decreased. However, the results obtained do not reveal what type of cells could serve the best for the transplantation. The results of different investigators show the effective decrease of the wound area only after repeated transplantation of MSCs, depending on the amount of the transplanted cells (Vojtassák et al., 2006; Falanga et al., 2007) and stimulated by local transplantation in collagen gel, but not after cell injection without extracellular matrix (Rustad et al., 2012; Shokrgozar et al., 2012). Our results show a decreased wound area in all cell treated groups in comparison with the control ones. The differences, detected in Lin^- cell treated groups using different extracellular components, allow assuming their role for transplanted cell activity in the wounded tissue.

An impaired transplanted cell adhesion in the injured tissue is rather a frequent problem. The absence of appropriate cell interactions with the environment after the transplantation results in the cell apoptosis or their migration from the injury site; thus, escaping the participation in the tissue regeneration process (Wong et al., 2013). In this study, prior to transplantation, the Lin^- cells were stained with the PKH26 fluorescent dye that is applied for long-time cell observation at *in vivo* conditions. By fluorescent microscopy was detected that different extracellular components, used for Lin^- cell

injection, influence the duration of cell survival and their distribution in the healing tissue (Fig. 5).

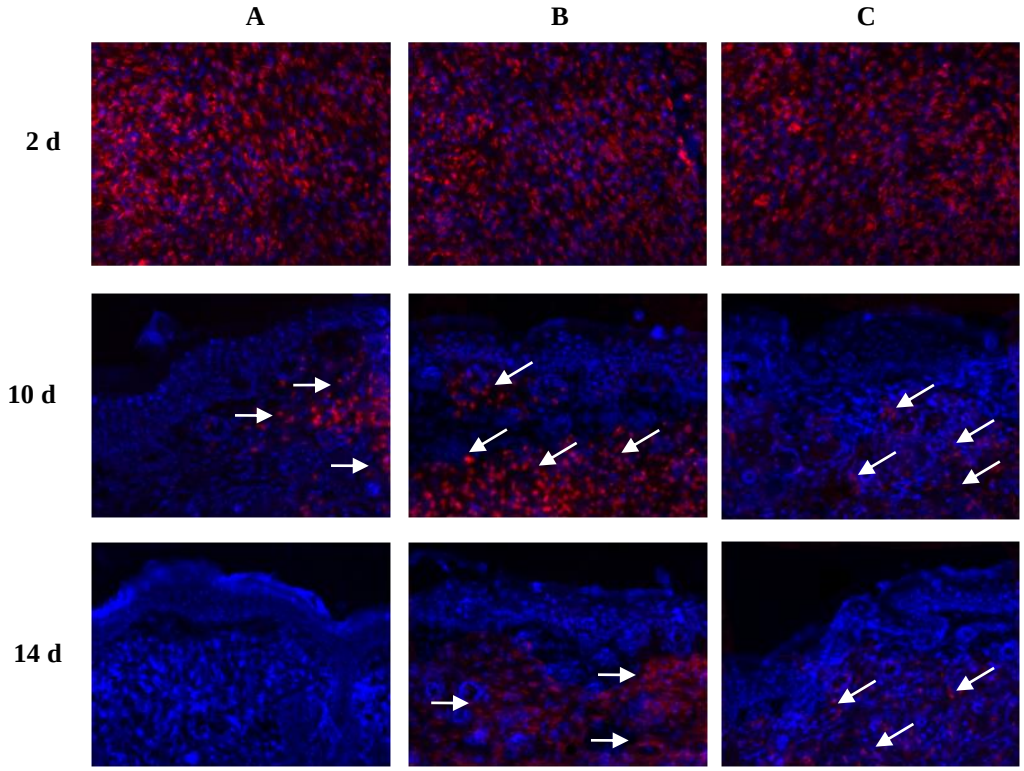

Fig. 5. Integration of transplanted Lin$^-$ cells into the wound. Lin$^-$ cells (1×10^6) were injected into the wound with PBS (A), type I collagen gel (B), and basement membrane matrix (C). Lin$^-$ cells were labeled with PKH26 (red, indicated by arrows) before transplantation; sections were counterstained with DAPI (blue); ×100.

Data of other investigators show that the MSCs, transplanted without a supplementary matrix, are not detected at the early stages of tissue regeneration (Lam et al., 2013). In our experiment, the labeled cells were detected in histological samples till day 10. In later time course, the biopsy samples from the healing tissue did not express Lin$^-$ cell population in the test group treated with PBS-suspended cells (Fig. 5A). However, when Lin$^-$ cells were injected in combination with type I collagen gel or basement membrane matrix, the presence of labeled cells in the healing tissue was registered till the last day of the experiment (Fig. 5B and C). This proves the significance of the supplementary biomaterials to ensure Lin$^-$ cell integration and survival in the injured tissue. The basement membrane components and type I collagen are suitable substrates for cell adhesion and also positively influence their integration in the foreign microenvironment.

Wound healing is a continuous process, divided into three main overlapping and gradually replacing each other phases – inflammation, proliferation and remodeling (Broughton et al., 2006). Each stage could be defined by specific reactions, where different cells, responsible for the inflammation control, the restoration of epidermal barrier, the formation of granulation tissue and the synthesis of extracellular matrix components could influence the structural and functional properties of the restored tissue (Reinke and Sorg, 2012). The influence of bone marrow-derived cells during different phases of wound healing can be direct or indirect (Wu et al., 2010). The analysis of the healing tissue after Lin^- cell transplantation was performed by the histochemical assays and quantified by a real-time PCR.

The results obtained show that following the first day after the skin injury, the inflammation takes place in all groups tested. The weakest inflammation was found in the group treated with Lin^- cells, transplanted in type I collagen gel. Data in literature reveal that one of the positive factors, influencing the use of stem cells for the injured tissue repair, is their anti-inflammatory properties (Biziulevičienė et al., 2007). The anti-inflammatory effect of bone marrow cells was tested in different models. Some studies reveal the long-term anti-inflammatory properties of the MSCs and the significance of HKCs only in the initial inflammatory reactions (Schuettpelz and Link, 2013; Zhang et al., 2013). In our experiment, following three days after cell transplantation, contrary to the control ones, histological analysis showed the initial phase of proliferation (Fig. 6). The phase of proliferation is characterized by the formation of granulation tissue, angiogenesis and epithelization. Started at the initial wound healing stages, undisturbed re-epithelization ensures a successful skin tissue regeneration process (Braiman-Wiksman et al., 2007). In our study, at day 3 after the induced skin injury, the beginning of the re-epithelization process in all cell injected groups was noticed (Fig. 6). In groups, treated with Lin^- cells in the basement membrane component gel or PBS solution, hyperproliferative epidermis was noticed on the wound edges that could be accounted for the increased proliferation of cells, participating in the process of re-epithelization (Fig. 6B and F). In the group, where the injured skin surface was treated with Lin^- cells, transplanted in type I collagen gel, the forming epidermis was found evenly distributed on the closed wound surface and was affected by more intense cell migration during the process of re-epithelization (Fig. 6D).

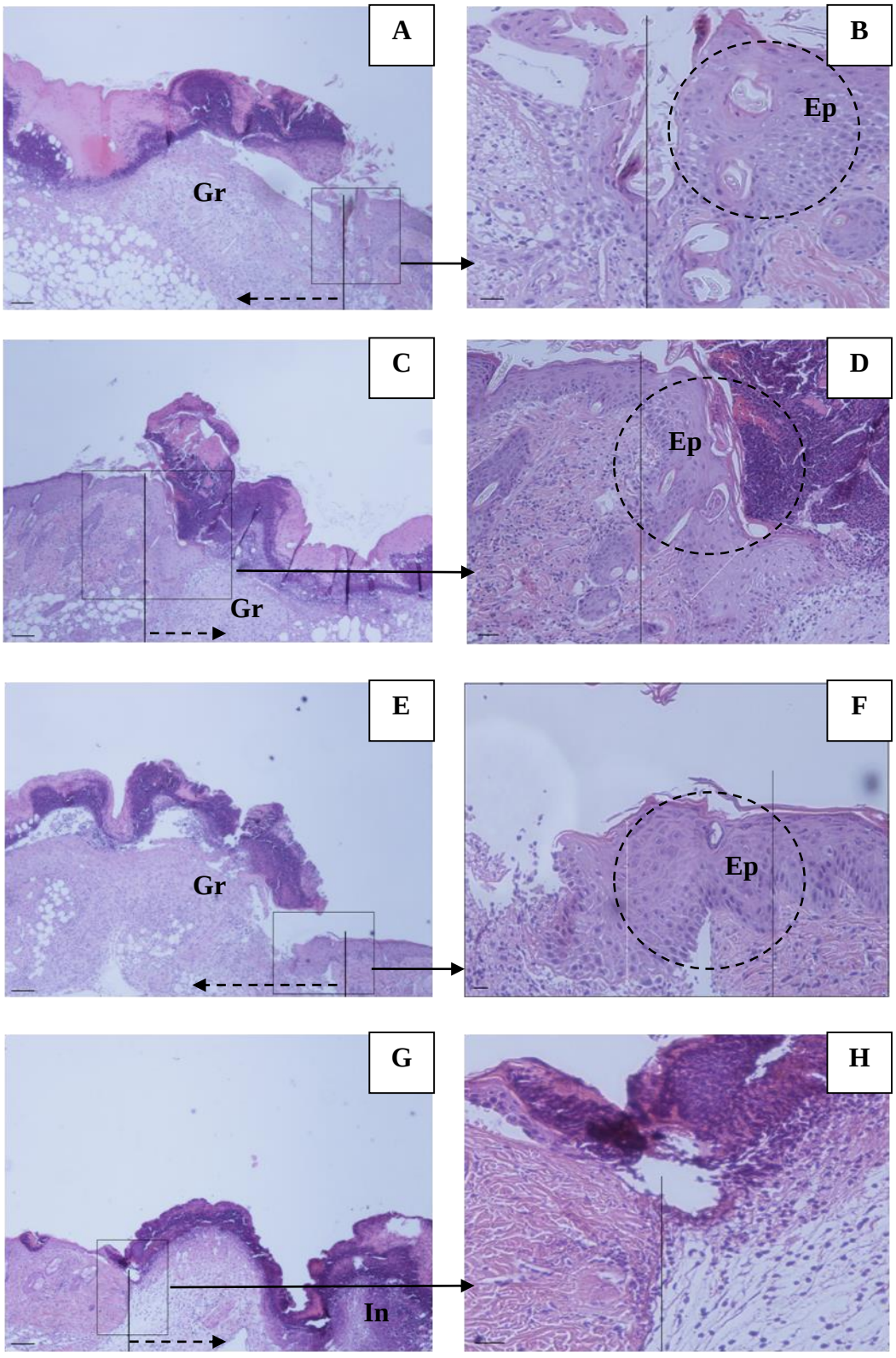

Fig. 6. Histologic examination of wound healing at day 3. Lin^- cells (1×10^6) were injected into the wound with PBS (A, B), type I collagen gel (C, D), and basement membrane matrix (E, F). Control group received no cells (G, H). H&E, ×40 (left) and ×100 (right). Abbreviations: De – dermis; Ep – epidermis; Gr – granulation tissue; In – infiltration of inflammatory cells. Dashed arrows indicate wound area; dashed circles indicate edge of migrating epithelium.

According to the data in literature, locally transplanted bone marrow MSCs influence twice faster wound closing by new epidermis in acute and chronic wounds (Mishra et al., 2012; Skardal et al., 2012). In the experiments of other investigators, where mouse skin wounds were treated with the adipose tissue stem cells, integrated in the complex collagen and silicone membrane, the process of re-epithelization was found to be delayed. During the first week after the cell transplantation, an inhibition of keratinocyte migration was noticed. These experiments revealed an increased (up to 1.5 times) re-epithelization only during the second week after transplantation (Nambu et al., 2009). In our study, the kinetics of the re-epithelization process *in vivo* was evaluated starting from day 3. The length of forming epidermis on the wound surface was measured. As compared with the control groups, a significant difference was found at all measurements in cell treated groups. The re-epithelization was earliest completed after Lin^- cell transplantation in type I collagen gel. In this group, the complete wound closure was seen at day 5 postwounding. However, in the groups, where Lin^- cells were transplanted in PBS solution or basement membrane component gel, new epidermis covered only 65.85±1.29% and 59.83±2.22%, respectively, of the wound surface. The re-epithelization process was completed in these groups only at day 7 of wound healing (Fig. 7).

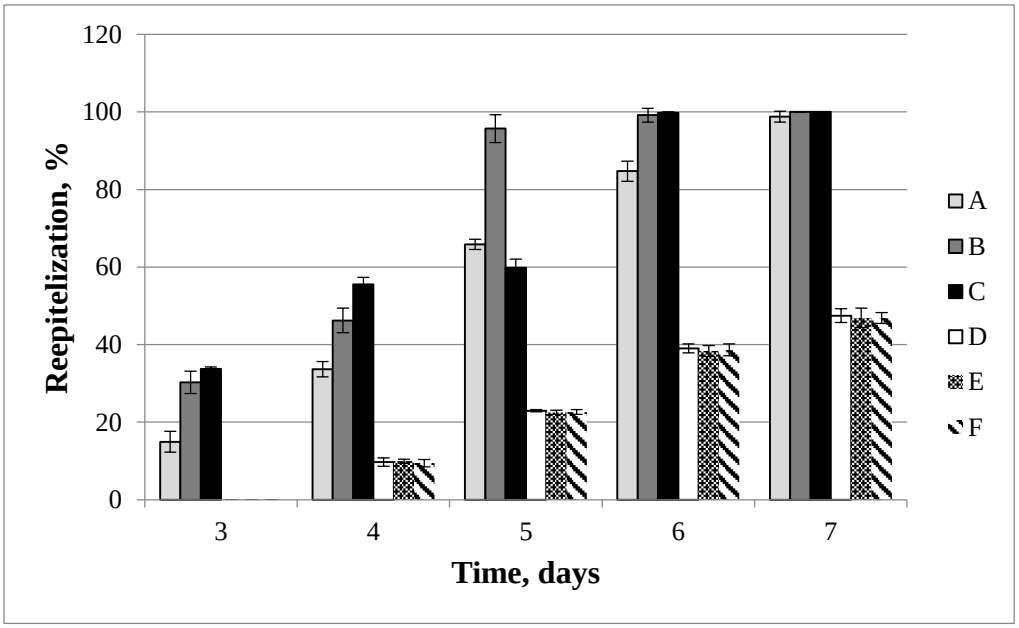

Fig. 7. Re-epithelization during wound healing. Lin^- cells (1×10^6) were injected into the wound with PBS (A), type I collagen gel (B), and basement membrane matrix (C). Control groups received PBS (D), type I collagen gel (E), and basement membrane matrix (F) without cells. Data are presented as mean ± SD (n = 3); $p < 0.05$.

Wound healing of the skin injury, where all tissue layers are lost, often ends in a partial restoration of tissue structure and functions. Such result is obtained because of the insufficient amount of cells, participating in the process of regeneration and lack of interactions between the different cell types and extracellular matrix components in the microenvironment of the injured tissue (Reinke and Song, 2012). In our experiments, at day 12, in all Lin^- cell treated groups, skin appendages were found in the dermis (Fig. 8A, B and C). However, in cell untreated groups, the granulation tissue at the injury site and a wide layer of undifferentiated epidermis define the still ongoing phase of proliferation (Fig. 8D). The presence and distribution of skin appendages in cell treated wounds were different depending on the matrix of transplanted cells. Different investigators using *in vivo* models showed the capability of stem cells at the site of injury to inhibit the infiltration of inflammatory cells and stimulate the formation of the granulation tissue (Shumakov et al., 2003); positively influence the process of re-epithelization (Nakagawa et a., 2005), to facilitate wound closure without scar formation (Mansilla et al., 2005), to stimulate the restoration of hair follicles and the formation of the functional tissue (Deng et al., 2005). There are data in literature stating that stem cells could influence the restoration of skin appendages only after the transplantation with the appropriate biomaterials (Huang et al., 2012) or after additional genetic manipulations prior to transplantation (Li et al., 2013). Our experiments show the distribution of separate structures in the peripheral part of the restored tissue and near the epidermis in the group treated with Lin^- cells, suspended in PBS solution (Fig 8A). Skin appendages, distributed over nearly the entire dermis, are found in the groups treated with Lin^- cells in type I collagen gel (Fig. 8B) or basement membrane component gel (Fig. 8C). However, in the latter group the distribution of restored structures is denser in the peripheral part of the wound in comparison to the central part. Skin appendages are distributed in the dermis according to different Lin^- cell integration at the site of injury after the transplantation. In all cell treated groups, the differentiated layer of epidermis, equivalent to the normal tissue, was formed at day 12.

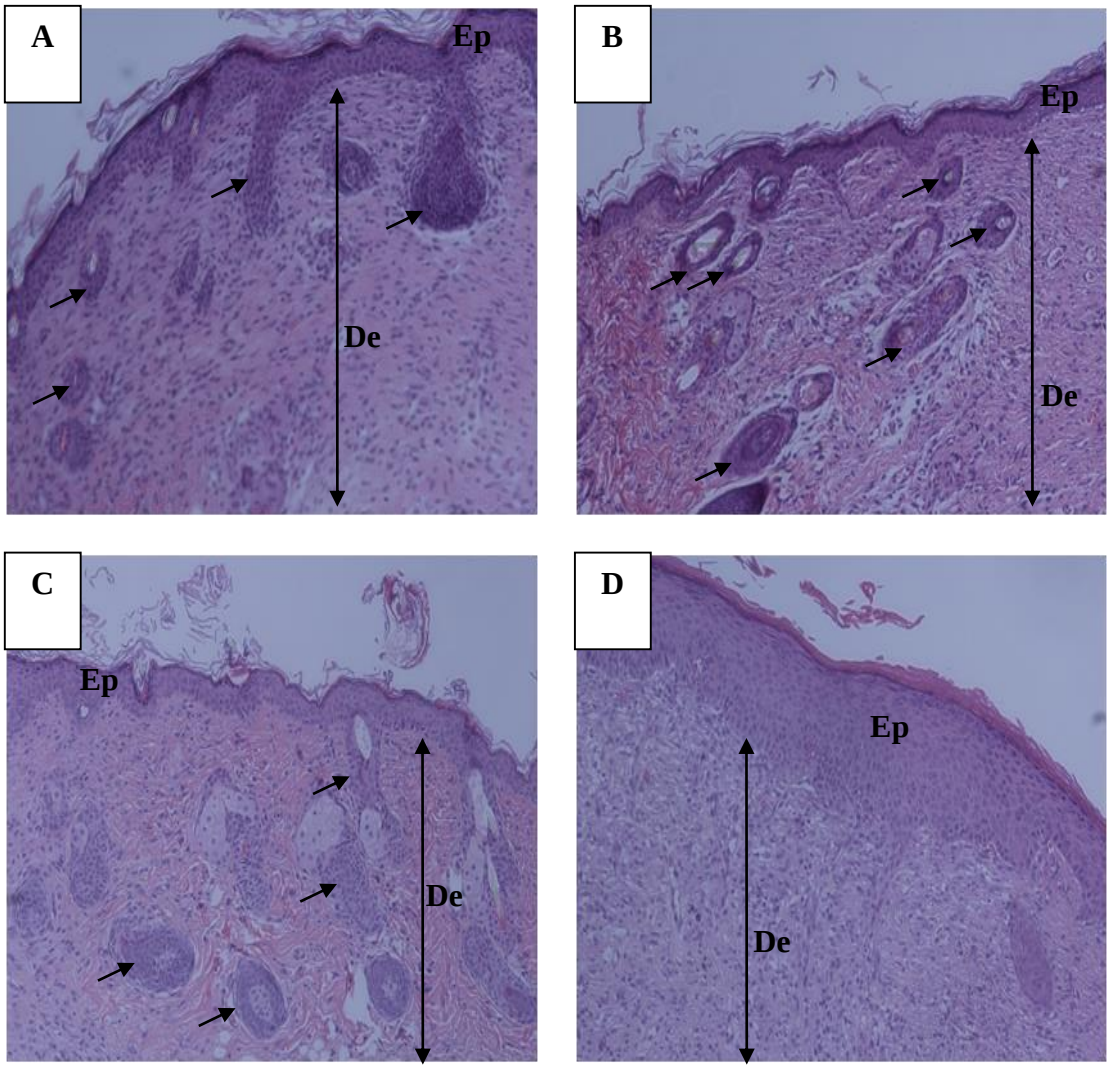

Fig. 8. Histologic examination of wound healing at day 12. Lin^- cells (1×10^6) were injected into the wound with PBS (A), type I collagen gel (B), and basement membrane matrix (C). Control group received no cells (D). H&E, ×100. Abbreviations: De – dermis; Ep – epidermis.

According to literature, stem cells can secrete several factors controlling the interaction of the microenvironmental molecules and tissue cells, influencing the proliferation and migration of the cells participating in the tissue regeneration process. They can also participate in the inflammation reactions and organization of extracellular matrix as well as stimulate the process of angiogenesis (Gnecchi et al., 2008; Baraniak and McDavid, 2010). We have examined the expression of cytokine genes in wound healing using real time PCR technique. The quantitative analysis showed that the gene expression of TGF-β, VEGF, IL-10 and TNF-α after Lin^- cell transplantation were significantly changed (Fig. 9).

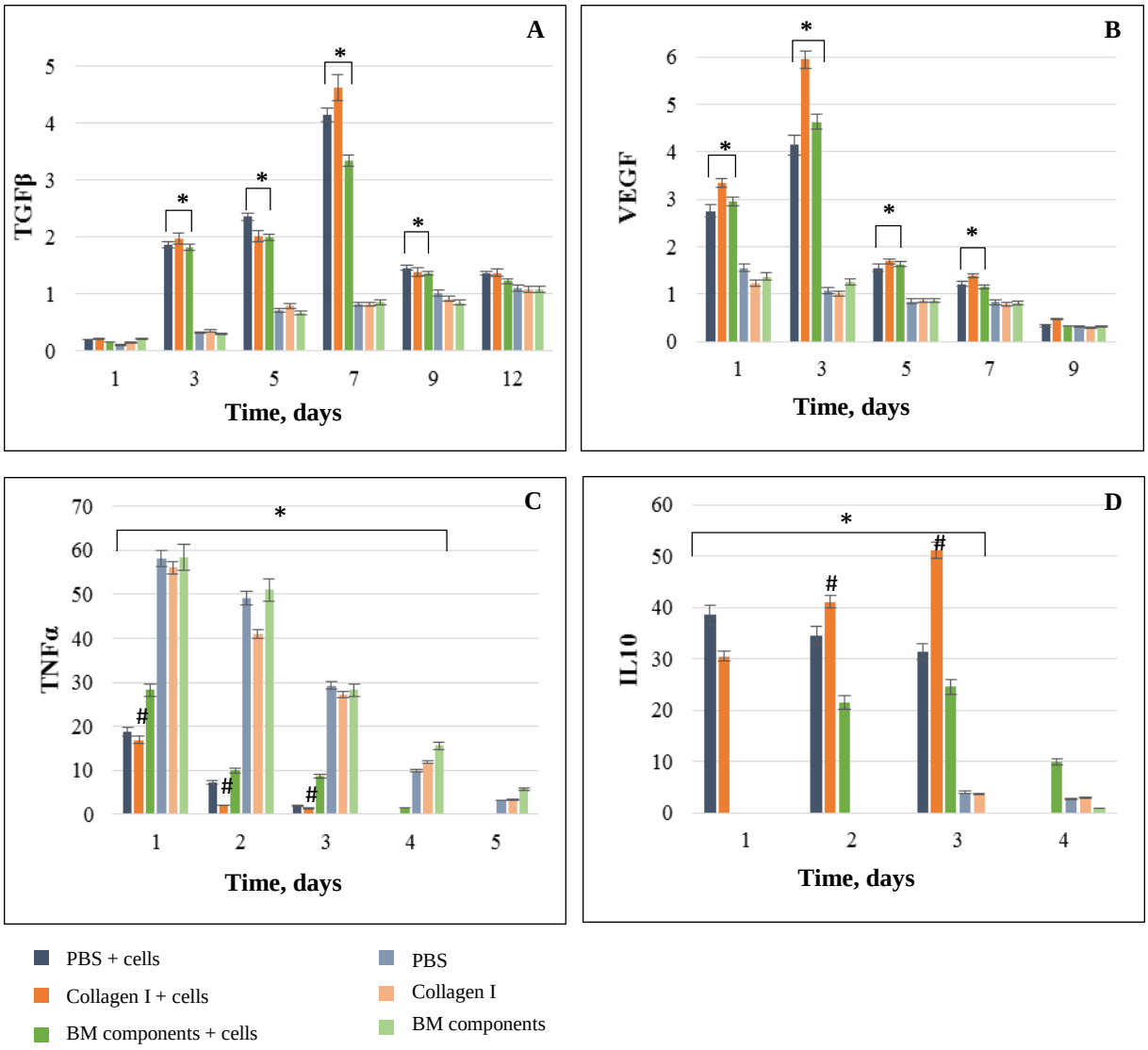

Fig. 9. Gene expression of cytokines during skin wound healing. TGF-β (A), VEGF (B), TNF-α (C) ir IL-10 (D) mRNA was quantified by real-time PCR. Results were demonstrated as mRNA in the wounds relative to non-wounded skin. The relative levels of gene expression were calculated by reference to the β-actin in each sample using the cycle threshold (C_t) method and were expressed as arbitrary units. Data are presented as mean ± SD (n = 3); * $p < 0.05$ experimental groups vs. control groups; # $p < 0.05$ wounds after cell transplantation in collagen gel vs. wounds after cell transplantation in PBS or basement membrane matrix.

The data of different investigators show the change in the gene expression of TGF-β and VEGF at the different healing stages. Their results proved that the expression of TGF-β gene is controlled from the early till the late stage of injury reactions (Kwon et al., 2008; Wong and Crawford, 2013); whereas the VEGF is more expressed at the stage of proliferation during the development of angiogenesis (Rustad et al., 2012; Sukpat et al., 2013). Our data show a significant increase of the TGF-β mRNA expression at the end of the inflammatory stage that remained till the late stage of proliferation (Fig. 9A). The

expression of VEGF gene was significantly changed after cell transplantation from the early stages of inflammation till the partway of the proliferation stage (Fig. 9B). As compared to the control group, the expression of TGF-β and VEGF genes was found to be six times increased in the cell injected groups. Among the groups, where the Lin^- cells were transplanted in different biomaterials, no significant changes in the level of TGF-β were registered. In comparison with the results from the control group, the increased expression of this cytokine in cell treated wounds remained till day 10. The expression of TGF-β gene in these groups was found to be 1.5 and 5.9 times increased at days 9 and 3, respectively (Fig. 9A). After the cell transplantation in type I collagen gel, the significant difference (1.7 times) in the VEGF expression was registered at day 3, as compared with the results, obtained from Lin^- cells in PBS solution or basement membrane component gel treated groups. During the development of the proliferation phase, the expression of VEGF mRNA in cell treated groups was found to be decreased but, nevertheless, it was higher than that in the control group. The relative expression of this cytokine, found after cell transplantation, was from two times at day 5 to 1.7 times at day 7 greater than in the cell untreated groups (Fig. 9B). At the initial stages of wound healing, it is very important to maintain the balance between the inflammatory and anti-inflammatory cytokines in order to ensure timely starting and ending the process reactions and successful formation of the renewed tissue (Behm et al., 2012). The data in literature about the role that undifferentiated cells play in the inflammatory process are rather contradictory. Pedroso et al., using a mouse skin wound model, described a significant inhibition of the inflammatory TNF- α secretion in the early wound healing stages after the transplantation of HSC. However, no marked influence on the secretion of the inflammatory IL-10 was detected (Pedroso et al., 2011). Contradictory results were obtained by another group of investigators who found out that the anti-inflammatory properties of umbilical cord blood HSC and the increased secretion of IL-10 amount in the healing tissue are connected (Kanji et al., 2014). In our study, the gene expression of TNF-α and IL-10 cytokines significantly differed in all groups until days 5 and 4. After the cell transplantation in type I collagen gel at day 2, the level of TNF-α mRNA was 22 times decreased as compared with the control groups and 5 times decreased as compared with the other cell treated groups (Fig. 9C). Up to day 4, the gene expression of IL-10 in the wounded tissue after treatment with the Lin^- cell populations in type I collagen gel was found to be twice

increased in comparison with the other cell treated groups and up to 13 times increased in comparison with the control groups (Fig. 9D).

To sum up the results, obtained in full-thickness skin wound model *in vivo*, we can assume that bone marrow Lin^- cell population possesses the properties to stimulate the tissue regeneration process. Type I collagen and basement membrane components, used for local transplantation, are not only suitable substrates for cell adhesion but also ensure their long-term integration into the injured site without the undesirable response to foreign substance. Histological analysis revealed the earliest inhibition of inflammation and the completion of re-epithelization process after transplantation of Lin^- cells in type I collagen gel, resulting in the restored tissue with the properties close to the native one. Such results might have been influenced by the interaction between the transplanted cells and collagen, as this protein is the main extracellular matrix component in the majority of tissues, thus maintaining the biological functions of different cell types. The ability to positively control the reactions, taking place during the wound healing process, gives the priority to the bone marrow Lin^- cell population to be used as a cell source for therapy. The results obtained add to the investigation of new advanced methods for complex wound healing.

Conclusions

1. Phenotypic analysis revealed that mouse bone marrow Lin^- cells express surface markers CD117, Sca-1, CD90, CD29 and CD113. It shows that Lin^- cell population is heterogeneous and contains various types of undifferentiated cells.
2. Skin extracellular matrix components enhance the regenerative properties of Lin^- cells *in vitro*. Cell proliferation was up to 1.5-fold higher in microenvironment containing basement membrane components, while the cell migration was more intensive using type I collagen.
3. Long-term integration of Lin^- cells into the wound area *in vivo* requires basement membrane components as well as type I collagen.
4. The qualitative analysis revealed that the most intensive regeneration process in the full-thickness skin wound model *in vivo* takes place after Lin^- cell transplantation in type I collagen gel. In this case, the inhibition of inflammation occurs earlier and the process of re-epithelization ends faster.
5. The quantitative analysis showed that Lin^- cells influence the gene expression of pro- and anti-inflammatory cytokines depending on the material used for transplantation. After cell transplantation in type I collagen gel, the expression of TNF-α mRNA decreases up to 5 times, and IL-10 mRNA expression increases up to 2 times. Early inhibition of inflammation is important for consecutive order of wound healing phases and ensures tissue regeneration process.
6. Transplanted Lin^- cells improve skin regeneration by influencing the gene expression of growth factors TGF-β and VEGF in different wound healing phases. Increased level of VEGF mRNA was established in inflammatory phase. The gene expression of TGF-β increased in the late inflammatory phase and remained till the beginning of maturation phase.

List of publications

1. **Ramanauskaitė G**., Kašėta V., Vaitkuvienė A., Biziulevičienė G. Skin regeneration with bone marrow-derived cell populations. *Int Immunopharmacol*. 2010; 10:1548-1551.
2. Biziulevičienė G., Kašėta V., **Ramanauskaitė G**., Vaitkuvienė A. Investigation of unmatured hematopoietic cell migration in a mouse model. *Cent Eur J Biol*. 2010; 5:585-589.
3. **Ramanauskaitė G**., Žalalytė D., Kašėta V., Vaitkuvienė A., Kalėdienė L., Biziulevičienė G. Skin extracellular matrix components accelerate regenerative potential of Lin^- cells. *Cent Eur J Biol*. 2014; 9:367-373.

Conference reports

1. **Ramanauskaitė G**., Kašėta V., Vaitkuvienė A., Biziulevičienė G. Skin regeneration with bone marrow-derived cell populations. 40th Annual Meeting of German Society for Immunology, Leipcigas, Vokietija. 2010, rugsėjo 22-25d.
2. **Ramanauskaitė G**., Vaitkuvienė A., Kašėta V., Biziulevičienė G. Integration of stem cell populations into biomaterials and their application for skin tissue regeneration. *Reg Med. Conference Abstract: World Conference on Regenerative Medicine.* 2011; 6(6s2):S232.
3. **Ramanauskaitė G**., Žalalytė D., Vaitkuvienė A., Kašėta V., Biziulevičienė G. Investigation of mouse bone marrow Lin^- cells regenerative properties *in vitro* and *in vivo*. *Front Immunol. Conference Abstract: 15th International Congress of Immunology (ICI).* doi: 10.3389/conf.fimmu.2013.02.00264.
4. **Ramanauskaitė G**., Vaitkuvienė A., Kašėta V., Žalalytė D., Kalėdienė L., Biziulevičienė G. Wound healing effect of bone marrow-derived stem/progenitor cell *in vivo*. *Reg Med. Conference Abstract: World Conference on Regenerative Medicine.* 2013; 8(6s):S381.

Reziumė

Sutrikus normaliam regeneraciją užtikrinančių reakcijų vystymuisi, pažeisto audinio atsistatymas baigiasi randinio audinio susiformavimu, kuris nepasižymi natyviam audiniui būdingomis struktūrinėmis ir funkcinėmis savybėmis. Šiuo metu klinikinėje praktikoje negyjančių žaizdų gydymui naudojami metodai suteikia laikiną apsauginę funkciją, padedančią užkirsti kelią infekcijoms bei dehidratacijai, tačiau neskatina audinio regeneracijos, todėl susiformavusiame audinyje trūksta pagalbinių struktūrų, užtikrinančių visišką funkcijų atsistatymą. Kamieninių ląstelių panaudojimas sutrikusio gijimo odos žaizdoms regeneruoti yra perspektyvus dėl unikalių šių ląstelių savybių nuolat atsinaujinti ir diferencijuotis į keleto tipų subrendusias ląsteles. Prieinamo šaltinio, kuriame būtų pakankamas kiekis ląstelių, bei tinkamų biomedžiagų, kurios užtikrintų efektyvią ląstelių transplantaciją, pasirinkimas yra pagrindinės užduotys, reikalaujančios intensyvių tyrimų, norint saugiai, sėkmingai ir patikimai taikyti kamieninių ląstelių terapiją. Šio darbo tikslas buvo įvertinti pelių kaulų čiulpų Lin^- populiacijos ląstelių įtaką odos audinio regeneracijai *in vivo*, taikant BALB/c linijos pelių visų odos sluoksnių pažeidimo modelį.

Darbo metu, magnetiniu ląstelių atskyrimo metodu iš bendros pelių kaulų čiulpų populiacijos buvo išgrynintos Lin^- ląstelės. Fenotipinė analizė parodė, kad šioje populiacijoje 81.89±2.92% ląstelių ekspresuoja CD117 paviršiaus žymenį, 16.46±3.51% – Sca-1, 9.48±2.93% – CD90, 5.53±1.8% – CD29, 6.64±0.93% – CD113. Visi šie žymenys būdingi nediferencijuotoms ląstelėms.

Ląstelių proliferacija bei migracija priklauso nuo sąveikų su tarpląstelinės medžiagos komponentais ir yra svarbūs procesai, kurie lemia audinio regeneracijos sėkmę. Atskirai proliferacijos ir migracijos procesus tirti *in vivo* sąlygomis yra sudėtinga dėl dugybės vienu metu vykstančių persidengiančių reakcijų. Ląstelių proliferacijos ir migracijos modeliavimas *in vitro* suteikia vertingų žinių ruošiant ląsteles transplantacijai *in vivo*. Šiame darbe įvertinome, kaip Lin^- ląstelių regeneracinės savybės priklauso nuo odos audinio tarpląstelinei medžiagai būdingų komponentų *in vitro*. Tyrimams pasirinkome I tipo kolageną ir pamatinės membranos komponentų (laminino, IV tipo kolageno, entaktino bei heparino sulfato) mišinį. Nustatėme, kad pamatinės membranos komponentų mišinys lemia iki 1.5 karto didesnį ląstelių proliferacijos aktyvumą, o I tipo kolagenas patikimai skatina ląstelių migracines savybes. Abi medžiagos buvo panaudotos lokaliai Lin^- ląstelių transplantacijai į visų odos sluoksnių pažeidimus *in vivo*.

Odos žaizdų gijimas yra nenutrūkstamas procesas, tačiau jo apibūdinimui išskiriamos trys viena kitą palaipsniui pakeičiančios stadijos – uždegimo, proliferacijos ir remodeliacijos. Kiekvienoje gijimo stadijoje vyksta specifinės reakcijos, kurios lemia atsinaujinusio audinio struktūrines bei funkcines savybes. Mūsų tyrime buvo atliekama lokali Lin^- ląstelių, suspenduotų PBS tirpale, I tipo kolageno arba pamatinės membranos komponentų geliuose, transplanatcija į visų odos sluoksnių pažeidimą, sukeltą BALB/c linijos pelėse. Nustatėme, kad ląstelės, transplantuotos I tipo kolageno gelyje, lemia ankstyviausią žaizdos sugijimą.

Odos žaizdų gijimo efektyvumą lemia transplantuotų ląstelių integracija pažeidimo židinyje. Dažna problema ląstelių terapijoje yra silpnas transplantuotų ląstelių įsitvirtinimas pažeistame audinyje, todėl ląstelės apoptuoja arba migruoja iš pažeidimo židinio ir nedalyvauja audinio regeneracijoje. Mūsų darbe buvo vertinama Lin^- ląstelių integracija gyjančiame odos audinyje. Nustatėme, kad PBS tirpale transplantuotos ląstelės, pažeidimo židinyje išlieka iki 10-osios paros. Transplantacijai panaudoję I tipo kolageno arba pamatinės membranos komponentų gelius, Lin^- ląstelių pažeidimo židinyje aptikome visą stebėjimo laiką.

Pasirinktais laiko momentais paėmę gyjančio audinio biopsijas, įvertinome, kaip transplantuotos Lin^- ląstelės veikia specifinius skirtingose gijimo stadijose vykstančius procesus. Buvo atlikta histologinė ir realaus laiko PGR analizė. Literatūros duomenimis, kamieninės ląstelės regeneraciją gali veikti tiesioginės diferenciacijos į reikiamus subrendusių ląstelių tipus būdu arba, išskirdamos citokinus bei augimo veiksnius, netiesiogiai kontroliuoti mikroaplinkoje esančių ir gijime dalyvaujančių ląstelių aktyvumą. Nustatėme, kad po Lin^- ląstelių transplantacijos I tipo kolageno gelyje, pažeistame audinyje uždegiminių ląstelių infiltracija sumažėja jau po 1-osios paros. Iki 3-iosios paros sumažėjęs uždegimas nustatytas po Lin^- ląstelių transplantacijos PBS tirpale bei pamatinės membranos komponentų gelyje. Kontrolinių grupių pelių žaizdose uždegiminių ląstelių aptikome iki 7-osios stebėjimo paros. Atlikę reepitelizacijos proceso kinetinę analizę, nustatėme, kad po Lin^- ląstelių transplantacijos žaizdos nauju epidermiu pasidengia iki 7-osios paros. Didžiausias reikšmingas skirtumas tarp grupių, kuriose pelėms buvo injekuojamos Lin^- ląstelės skirtingose medžiagose, nustatytas iki 5-osios stebėjimo paros. Į žaizdas transplantuojant ląsteles su I tipo kolageno geliu, 5-ąją dieną reepitelizacijos procesas buvo baigtas. Šiuo metu naujas epidermis po Lin^- ląstelių

transplantacijos su pamatinės membranos komponentų geliu arba PBS tirpalu dengė atitinkamai 59.83±2.22% ir 65.85±1.29% žaizdų paviršiaus. Kontrolinėse grupėse žaizdos nauju epidermiu pasidengė iki 11-osios stebėjimo paros. Po Lin^- ląstelių transplantacijos atsinaujinusio audinio dermoje 12-ąją parą nustatytos susiformavusios pagalbinės struktūros, kurių išsidėstymas priklausė nuo transplantacijai pasirinktos medžiagos. Lyginant su sveiku audiniu, panašus pagalbinių struktūrų išsidėstymas visame dermos sluoksnyje nustatytas po ląstelių transplantacijos I tipo kolageno gelyje. Po Lin^- ląstelių transplantacijos PBS tirpale arba pamatinės membranos komponentų gelyje, didžioji dalis atsinaujinusių struktūrų išsidėsčiusios periferinėje susiformavusios dermos dalyje ir po epidermiu. Kiekybinė analizė parodė, kad po Lin^- ląstelių transplantacijos gyjančiame odos audinyje reikšmingai pakinta citokinų TGF-β, VEGF, IL-10 ir TNF-α genų raiška. Lyginant su kontrolinėmis grupėmis, po ląstelių transplantacijos nustatyta iki 6 kartų didesnė TGF-β ir VEGF genų raiška 3-iąją tyrimo dieną. Tarp grupių, kuriose pelėms į žaizdas Lin^- ląstelės buvo transplantuojamos skirtingose medžiagose, patikimų TGF-β mRNR raiškos skirtumų nenustatyta. Po ląstelių transplantacijos kolageno gelyje VEGF mRNR raiška patikimai skyrėsi (1.7 karto) 3-iąją dieną, lyginant su grupėmis, kuriose Lin^- populiacijos ląstelės buvo injekuojamos PBS tirpale arba pamatinės membranos komponentų gelyje. Citokinų TNF-α ir IL-10 genų raiška patikimai skyrėsi visose grupėse atitinkamai iki 5-osios ir 4-osios dienų. Po ląstelių transplantacijos kolageno gelyje 2-ąją dieną TNF-α mRNR raiška buvo iki 22 kartų mažesnė nei kontrolinėse grupėse ir iki 5 kartų mažesnė nei po ląstelių transplanatcijos PBS tirpale arba pamatinės membranos komponentų gelyje. Iki 4-osios dienos, IL-10 mRNR raiška pažeistame audinyje po Lin^- ląstelių transplantacijos kolageno gelyje buvo iki 2 kartų didesnė nei ląsteles transplantuojant PBS tirpale arba pamatinės membranos komponentų gelyje ir iki 13 kartų didesnė nei kontrolinėse grupėse.

Mūsų eksperimentų rezultatai parodė, kad kaulų čiulpų Lin^- ląstelių populiacija skatina pažeisto odos audinio regeneraciją. Atlikę palyginamuosius tyrimus, nustatėme, kad šių ląstelių poveikis priklauso nuo lokaliai transplantacijai pasirinktų medžiagų. Gauti rezultatai svarbūs sutrikusio gijimo žaizdų gydymo metodų vystymui.

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Acknowledgements

I would like to express my greatest gratitude to my supervisor prof., dr. Genė Biziulevičienė for giving me the opportunity to be a scientist. Thanks for her great guidance and assistance, stimulating suggestions and extraordinary patience throughout this long journey.

I would also like to express my sincere gratitude to my colleagues. Many thanks for dr. Vytautas Kašėta and dr. Aida Vaitkuvienė for their significant involvement in the preparation of dissertation. I am particularly grateful for the assistance in experiments given by Dovilė Žalalytė. I thank the many others who participated in my scientific journey. I cannot name them all here, but their support has been invaluable, at a time when it was especially needed.

And last but not least, I would like to thank my family. I am grateful for their patience and constant support, without them this thesis would not have been possible.