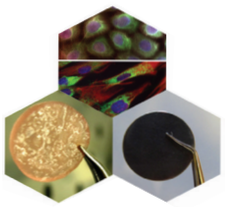

CELL AND TISSUE ENGINEERING LABORATORY

***Irina Titorencu**, PhD*
HEAD OF LABORATORY

STAFF

***Ana Maria Roșca,** PhD*
***Vasile Pruna,** PhD*
***Raluca Țuțuianu,** PhD Student*
***Maria Cristina Mihai (Coroţchi)**, PhD Student*
***Mădălina Daniela Iacomi,** Master Student*
***Gabriela Florea,** Master Student*
***Luminița Rădulescu,** Master Student*
***Constanța Stan,** Technical Assistant*
***Roxana Vlădulescu,** Technical Assistant*
***Irina Raluca Toader,** Technical Assistant*

FORMER RESEARCH STAFF:

***Victor V. Jinga,** PhD /Head of the Department: 1979-2017/*
Ioana Manolescu, Technical assistant
Camelia Elena Matei, Technical assistant

CORE LABORATORY UNIT
Cell Culture Facility
responsible
Irina Titorencu

CELL AND TISSUE ENGINEERING LABORATORY

Irina Titorencu, *PhD*

Head of Laboratory
E-mail: *irina.titorencu@icbp.ro*

Major position /appointments and professional training

- Scientific researcher II; Principal investigator
- Advisor students master program
- Invited peer reviewer for international journals
- Visiting scientist: Centre for Vision and Vascular Science, Queen's University, Belfast, UK; University of Chemical Technology and Metallurgy, Sofia, Bulgaria
- Member of the Scientific Council of ICBP

PUBLICATIONS

Between 2001 and 2019 the former and current scientists of the laboratory published 47 scientific papers and 5 chapters in monographs.

MAJOR RESEARCH INTERESTS

- **Isolation and characterization of human mesenchymal adult stromal cells (MSC);**
- **Differentiation of bone marrow-derived MSC into osteoprogenitor cells-osteoblasts.**
- **Biocompatibility assessment of materials proposed for bone and skin engineering**
- **The potential of MSC secretome in wound healing therapy and development of a wound model 3D organotypic culture for in vitro study of wound healing therapeutic approaches.**

TECHNICAL EXPERTISE

- **Cell culture techniques**
- **Biocompatibility assessment for organic and inorganic materials**
- **2D and 3D culture systems**
- **Histological processing and staining**
- **Ultracentrifugation techniques for exosomes isolation**
- **Fluorescence microscopy**
- **Molecular biology (Western blot, PCR, RT-PCR, qPCR)**

SELECTED NEW FINDINGS

• Under appropriate treatment, human bone marrow mesenchymal stromal cells (MSC) can differentiate into osteoblasts.

• BMP-4 loaded in 3D collagen-hyaluronic acid scaffolds has good physical-chemical and morphological properties, promotes adhesion, maintains viability and sustains the migration of osteoblast-like cells into scaffolds.

• TiO(2) nanotubes annealed samples with hydrophilic properties sustain the biocompatibility and osteogenic differentiation of human osteoprogenitor cells.

• Collagen nervous conductors are able to sustain peripheral nerve regeneration.

• MSC secretome improves the colonization of collagen 3D scaffolds, by supporting the adherence and proliferation of keratinocytes and by inducing a pro-healing phenotype in dermal fibroblasts.

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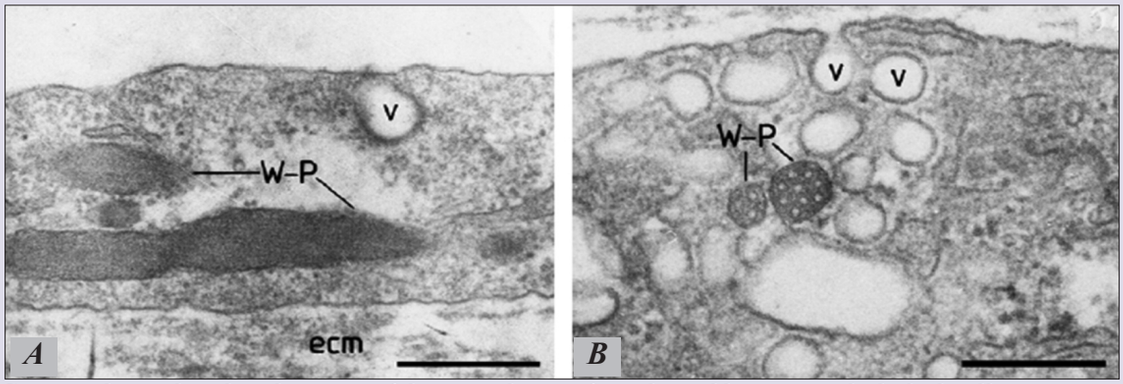
PREVIOUS PROJECTS

1. ESTABLISHMENT AND CHARACTERIZATION OF HUMAN CELL LINES

Pure vascular endothelial cell line from vascular placenta (HPEC) were isolated using an original procedure developed in our institute. The differentiation and the purity of the cell line was assessed by morphological studies, demonstration of Von Willebrand factor antigen, uptake of AcLDL-DiI, angiotensin converting enzyme activity and interactions with lectins. (V.V. Jinga et al., Placenta: 21(4): 325-336, 2000).

The cells were subsequently used for numerous projects of the institute.

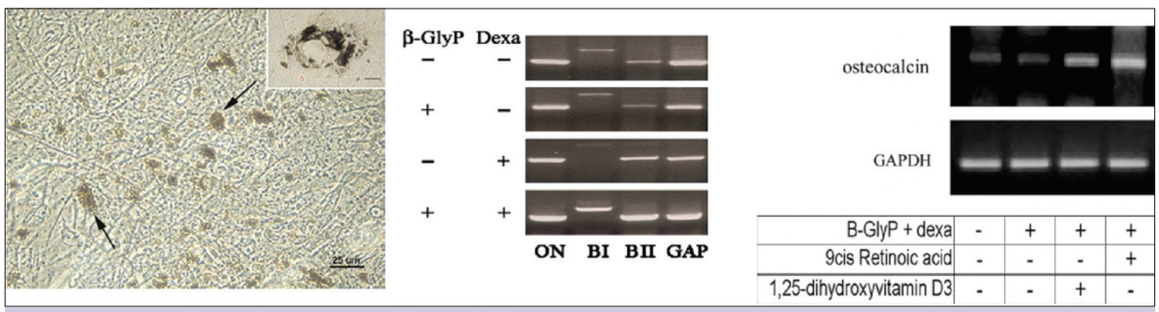


*Section through a distal region of the cytoplasm of a cultured human endothelial cell containing a numerous Weibel–Palade bodies that appear as long, rod-like shape, with tubular content (**A**), bar: 0.36 µm. (**B**) In cross-section, their microtubular internal substructure is better identified. W-P: Weibel–Palade body; v: vesicle; ecm: extracellular matrix. Bar: 0.21 µm.*

2. OSTEOBLASTS DIFFERENTIATION FROM HUMAN BONE MARROW MESENCHYMAL STROMAL CELLS (MSC)

Bone marrow (BM-MSC) were isolated from several donors and characterized in terms of their trilineage differentiation capacity with emphasis on the osteoblastic differentiation for traumatic bone injury therapies.

We reported that a subpopulation of human mesenchymal progenitor cells can be cultured *ex vivo*, and under proper stimuli (dexamethasone and β-glycerophosphate) display several osteogenic markers like osteonectin, bone sialoproteins I and II as well as osteocalcin (Titorencu et al., Cytotherapy: 9(7): 682-696, 2007).

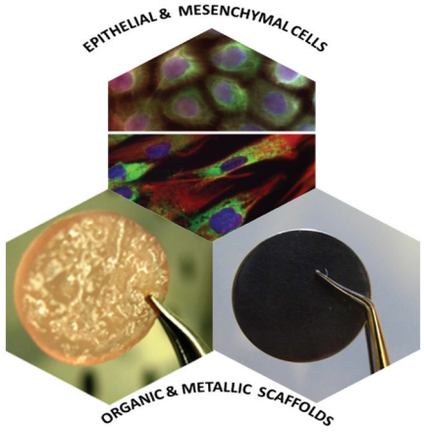


Bone marrow derived MSC are able to differentiate in vitro into osteoblasts: Von Kossa staining for extracellular calcium deposits on osteoprogenitor cells treated for 2 weeks with dexamethasone and β glycerophosphate (left), and increase the gene expression of osteonectin, bone sialoproteins I and II, and osteocalcin on bone marrow mesenchymal stromal cells as revealed by RT-PCR (right).

3. ASSESSING OF BIOCOMPATIBILITY OF NOVEL BIOMATERIALS WITH INNOVATIVE COATINGS FOR ORTHOPAEDIC APPLICATIONS AND BIODEGRADABLE SCAFFOLDS FOR SKIN REGENERATION

We tested the in vitro biocompatibility of organic matrices (based on type I collagen) as well as their combination with synthetic active principles on different human cell types: MSC, endothelial cell, human osteoblasts, skin cells (fibroblasts and keratinocytes).

Also, we evaluated the interaction between alloys with various inorganic coatings and human osteoprogenitor/osteoblasts cells.

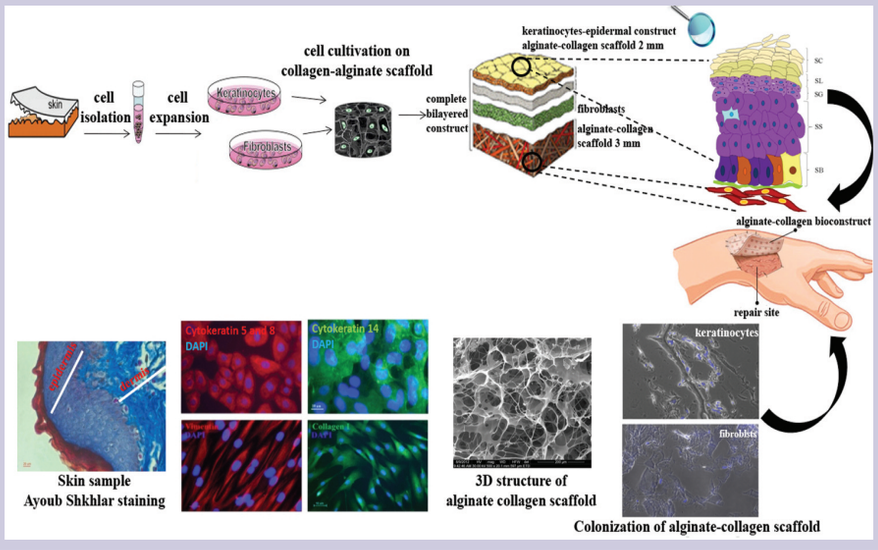


In vitro biocompatibility studies of epithelial and mesenchymal cells with organic and metallic scaffolds for tissue engineering.

4. DEVELOPMENT OF A DERMO-EPIDERMAL SKIN SUBSTITUTE SEEDED WITH HUMAN KERATINOCYTES AND DERMAL FIBROBLASTS.

Human keratinocytes and fibroblasts derived from healthy skin samples were isolated and expanded *in vitro*. Following their characterization for specific markers: keratin 5 and 14 for keratinocytes, and vimentin and type I collagen for dermal fibroblasts, the two type of cells were seeded on 3D collagen-alginate scaffolds.

The colonization and cell proliferation were followed via XTT analysis, DNA quantification and microscopy, in time, showing that these types of scaffold are suitable for wound repair. The experimental design and most significant result are schematically shown in the below Figure.



Schematic representation for the development of a dermo-epidermal skin substitute. SB - stratum basale, SS - stratum - spinosum, SG – stratum granulosum, SL – stratum lucidum, SC – stratum squamous

CURRENT PROJECTS

1. EVALUATION (*IN VITRO*) OF WOUND HEALING CAPACITY OF BM-MSC AND ADIPOSE DERIVED MSC

Chronic wounds - a quiet pathology affecting a large part of the diabetic patients, place a considerable burden on healthcare resources. MSC represent a promise in the care of such pathology.

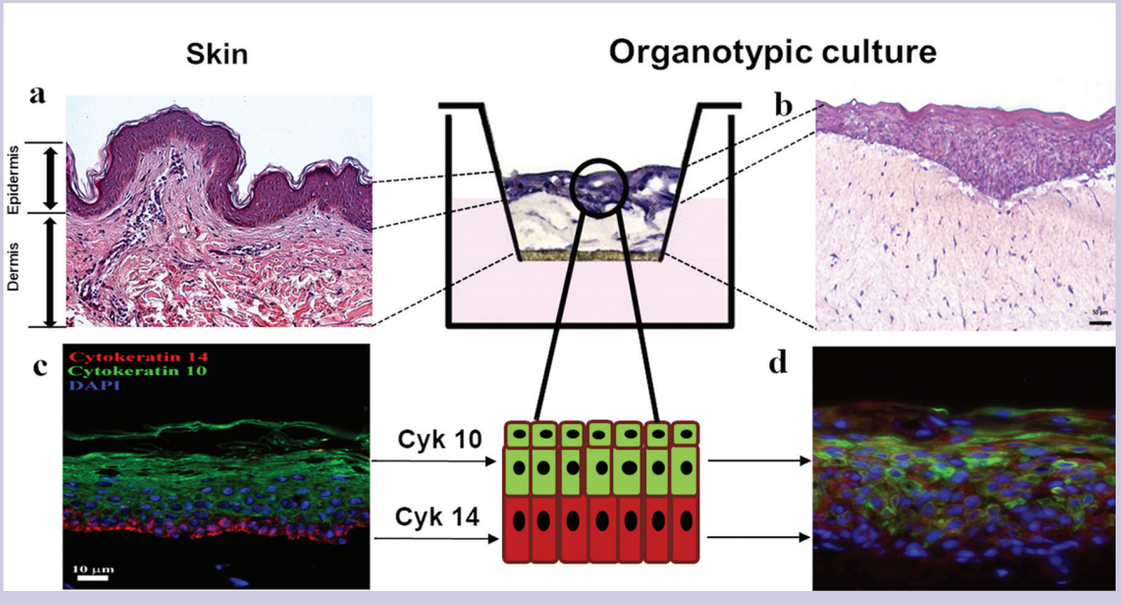
Our research is currently focused on two main strategies to improve the wound healing process by using human stromal mesenchymal stromal cells harvested from bone marrow and adipose tissue.

- **Contribution of MSC to the re-epithelialization process of the biomimetic skin cultures by differentiation into keratinocytes and by paracrine activity**

GOALS

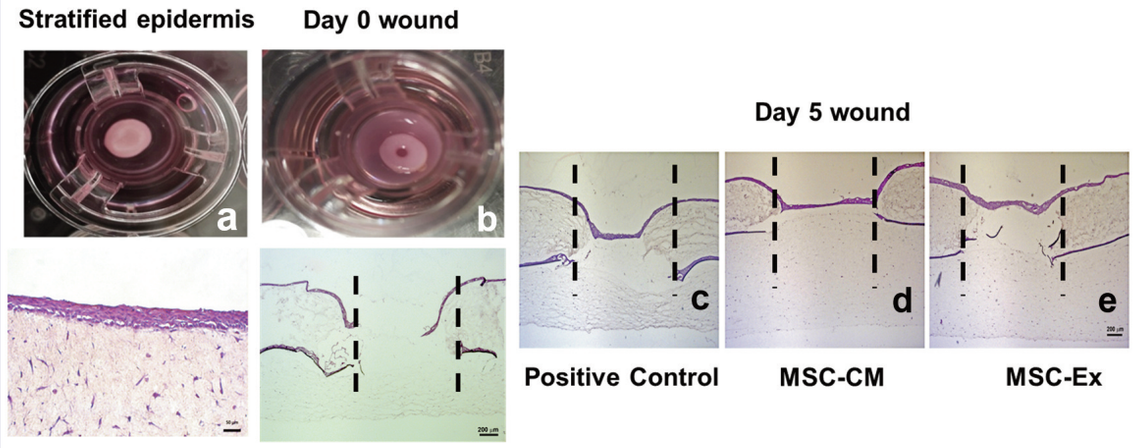
The project comparatively evaluates the re-epithelialisation capacity of MSC isolated from 2 sources: bone marrow and adipose tissue employing organotypic skin cultures kept in normal and high glucose (mimicking diabetes) conditions.

We established a 3D system consisting of human dermal fibroblasts (DFs) embedded in a gel-like matrix - the dermal component - and stratified human keratinocytes - the epidermal component. Compared to traditional "on-a-plastic" systems, this organotypic culture reproduces the three-dimensional stratified space within which skin cells normally live and function *in vivo*. Moreover, this *in vitro* model represents a cost-effective, time-efficient and ethical alternative to animal models.



Development of in vitro 3D organotypic skin culture:
a) H&E staining; b) Immunostaining of cytokeratin 14 (marker for basal layer) and cytokeratin 10 (marker for suprabasal layers) in skin (b) and organotypic culture (d).

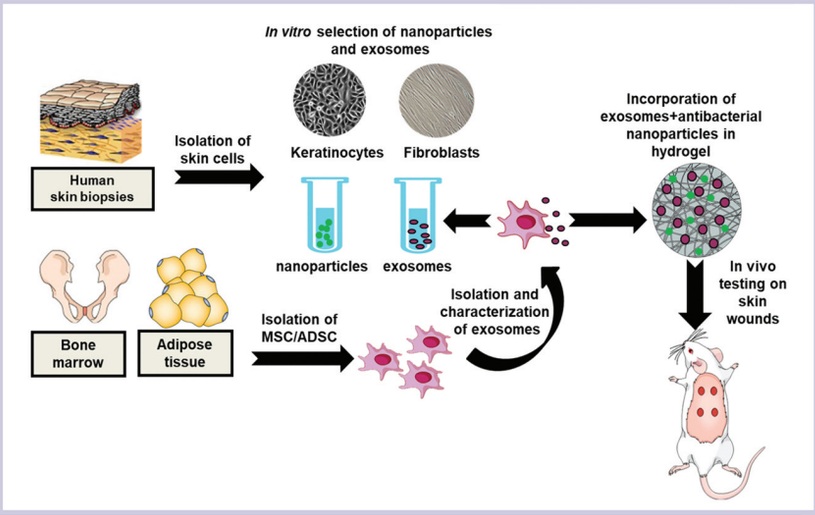
Using the organotypic culture, we established an in vitro wound healing model, which was further used in order to evaluate the regenerative properties of MSC-derived factors. We assessed the capacity of MSC-derived whole secretome and of isolated exosomes to support the migration of keratinocytes toward the wounded area.



The excisional in vitro wound model performed on skin organotypic cultures and the effect of MSC secretome and exosomes on the re-epithelialization process. Hematoxylin-eosin–stained sections showed punched wounds with clearly defined wound margins (denoted by broken lines. The re-epithelialization of the wound cultures was followed for 5 days. Compared with the positive control (complete medium with serum), the wound models maintained in MSC conditioned medium (MSC-CM) or exposed to exosomes (MSC-Ex) showed also complete re-epithelialization of the epidermal layer.

2. DEVELOPMENT OF TISSUE ENGINEERED CONSTRUCT WITH ANTIBACTERIAL PROPERTIES BASED ON FIBRIN, MSC DERIVED EXOSOMES AND NANOPARTICLES FOR THE TREATMENT OF CHRONIC WOUNDS

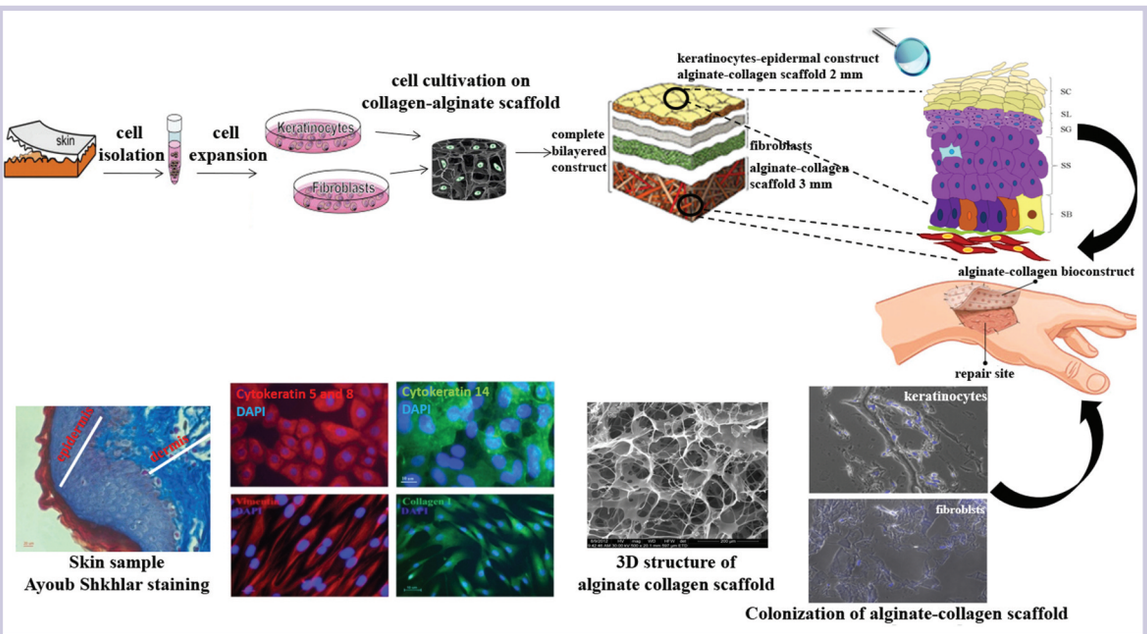
Within this project we use the curative and anti-inflammatory properties of MSC derived exosomes by developing a tissue engineered construct with antibacterial properties based on biodegradable organic polymers (fibrin), exosomes and antibacterial nanoparticles for the healing of chronic wounds. For this purpose, MSC derived from lipoaspirate and bone marrow are used to isolate the exosomes. The regenerative properties of these nanovesicles, as well as the effects of the antibacterial hybrid silver-chitosan nanoparticles are tested on cells isolated from human skin samples - keratinocytes and fibroblasts. After the selection of the most potent type of exosomes and nanoparticles, these will be incorporated in a fibrin-based hydrogel in order to be tested on mouse skin wound model.



Schematic representation of developing of a tissue engineered construct based on fibrin, MSC derived exosomes and nanoparticles for the treatment of chronic wounds.

• Isolation and characterization of mesenchymal stromal cells-derived exosomes

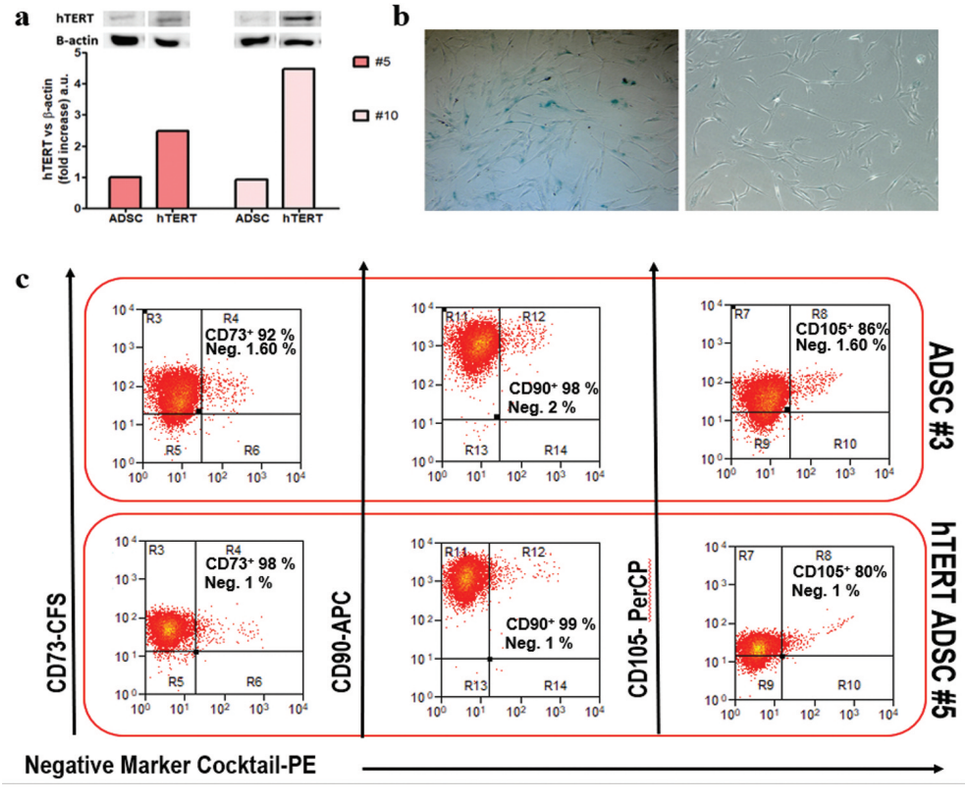
The exosomes, important mediators of the intercellular communication, are extracellular vesicles of 30-150 nm. MSC-derived exosomes were isolated were isolated from MSC conditioned medium (CM) by ultracentrifugation and characterized by Nanosizer analysis, flow cytometry and western blot. Thus, characterization of exosomes revealed the presence of CD9, CD63 and CD81 positive vesicles of ~ 125 nm diameter.



Characterization of MSC-derived exosomes.
(a) size evaluation by Nanosizer analysis; (b) Flow cytometry showing the expression of exosomal markers CD63 and CD90; (c) western blot showing the expression of CD81 and the lack of endoplasmic reticulum marker calnexin.

• Generation and characterization of an immortalized human adipose mesenchymal stromal cell line

Adipose-derived mesenchymal stromal cells (ADSCs) are a promising source for the cellular therapy due to their biological availability, multipotency, and rich secretome. However, because the limited life span of primary ADSCs during *in vitro* expansion impedes their use in clinical applications and basic research, we aimed to immortalize ADSCs by lentiviral transfection with human telomerase (hTERT). The lentivirus was produced by transfection of AD293 packaging cells using a third-generation lentiviral system (pMD2.G, pMDLg_pRRE, pRSV-Rev) and the hTERT codifying plasmid (pLV-hTERT-IRES-hygro). The immortalized cells were characterized by Western Blot (hTERT expression), flow cytometry and by assessing the multipotent differentiation capacity. A higher expression of hTERT in transfected cells comparing to the original ADSCs. The flow cytometry analysis showed that the surface markers pattern was characteristic for mesenchymal stromal cells: less than 2% hematopoietic markers (CD45, CD34, CD11b, CD79a, HLA-DR), over 90% positive for CD73, CD90 and CD105. The stem cells properties and potential to propagate in culture were maintained up to the 20th passage, while the original ADSCs stopped to multiply after the 10th passage. Moreover, the senescence assessment of ADSC versus hTERT ADSC by β-Galactosidase staining indicated the maintenance of proliferative properties.

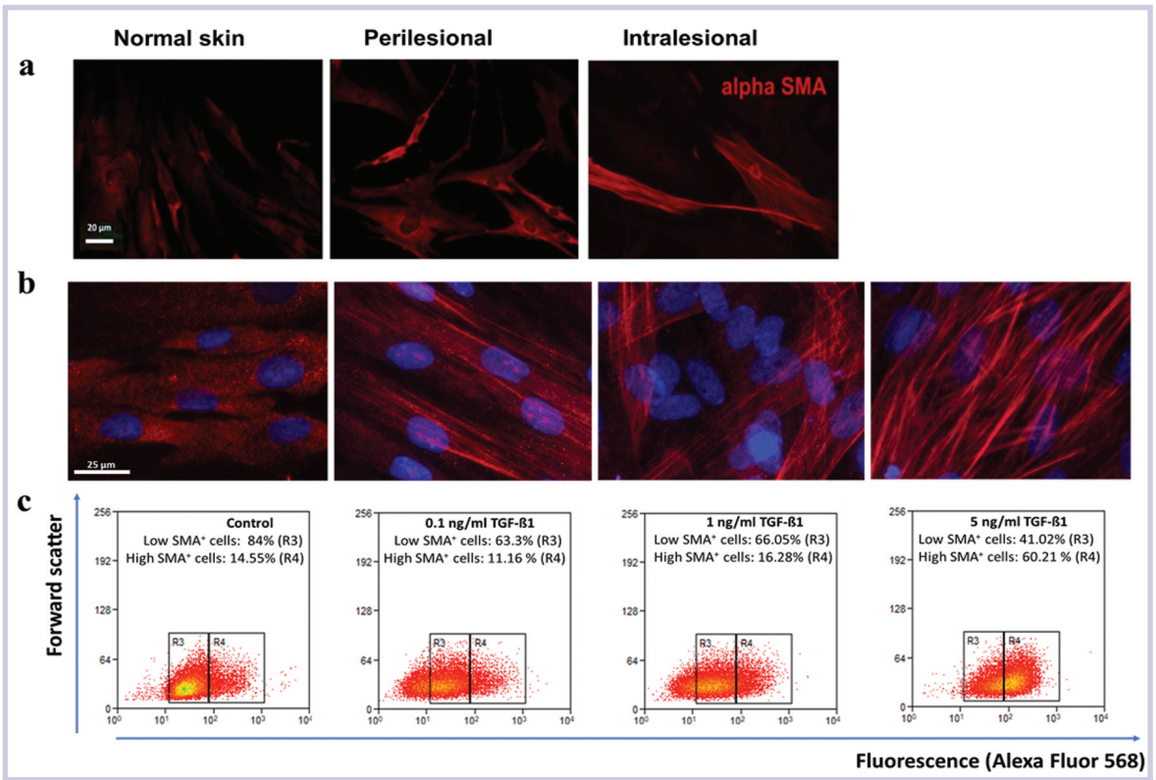


Characterization of hTERT ADSC

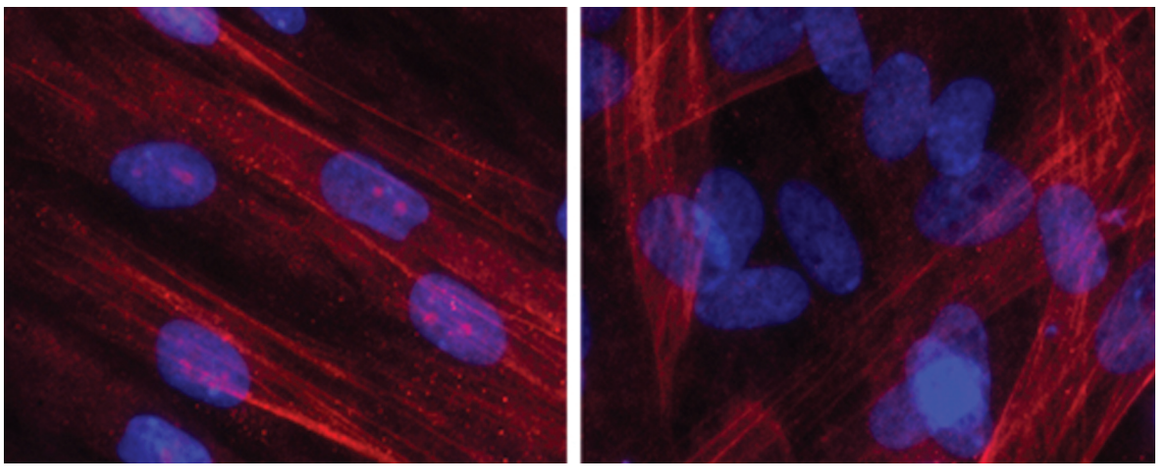
(a) Western blot analysis of hTERT protein expression levels in ADSC and hTERT ADSC; (b) Senescence assessment of ADSC versus hTERT ADSC by β-Galactosidase staining (blue); (c) Flow cytometry analysis of surface markers.

● Establishment of human dermal myofibroblasts lines

Myofibroblast differentiation and activation is a critical event in the pathogenesis of human fibrotic diseases and hypertrophic scars. Thus, we obtained cells with myofibroblasts phenotype by two methods: (i) isolation from hypertrophic scars and (ii) treatment with TGF-β1 of normal human dermal fibroblasts. These cells will be further used in order to assess the anti-fibrotic properties of MSC-derived secretome versus exosomes.



***Characterization of myofibroblasts**:*
(a) Immunocytochemistry showing the expression of α-SMA organized in stress fibers in fibroblasts isolated from hypertrophic scars; (b) and (c) characterization of the myofibroblasts phenotype induced by treatment with TGF-β1.



CELL AND TISSUE ENGINEERING LABORATORY

CURENT COLABORATIONS

• Carol Davila University of Medicine and Pharmacy Bucharest (Ioan Lascăr)

• University of Medicine and Pharmacy Craiova (Laurențiu Mogoanta)

• Emergency Hospital Floreasca Bucharest (Paul Neagu)

• Politehnica University of Bucharest (Alexandru Grumezescu)

ONGOING GRANTS AWARDED BY COMPETITION

• **2018-2020: TE project, cod PN-III-P1-1.1-TE-2016-1592 PROSKIN):** " *Mesenchymal stromal cells contribution to the reepithelialization process of the biomimetic skin cultures by differentiation and paracrine factors* " Project coordinator: Irina Titorencu - IBPC "N. Simionescu".

• **2018-2020: PCCDI project, cod PN-III-P1-1.2-PCCDI-2017-0749 (NANO-LIFE):** "*Bioactive Nanostructure for Innovative Therapeutic Strategies*" project coordinator: Prof. Dr.Laurentiu Mogoanta, University of Medicine and Pharmacy Craiova, Partners: IBPC "N. Simionescu", "CAROL DAVILA" University of Medicine and Pharmacy Bucharest., Politehnica Universtity of Bucharest

COMPLETED GRANTS AWARDED BY COMPETITION

CNCSIS: Osteoprogenitors cellular factors in treating deficiencies of bone consolidation. (2001 - 2003), project director Dr. Victor Jinga

VIASAN: Differentiation and proliferation of human osteoblasts in vitro, originating from marrow stromal stem cells; Their utilization as autologous progenitor transplant bone in orthopedic surgery (2001-2003), project director Dr. Victor Jinga

CNCSIS-TD - In vitro effect of the ischemic myocardium released factors in the cardiomyocyte differentiation of adult stem cells (2007-2009), project director Dr. A.M. Rosca

PD CNCSIS (PD 134/2010): Interrelation of human osteoprogenitor cells with human endothelial cells studied in vitro through bicameral models and three-dimensional media; initiation and promotion of angiogenesis. (2010 - 2012), project director Dr. Irina Titorencu

PN-II-PT-PCCA-2013-4-2267 : Biomagia - Biodegradable implants from magnesium alloys used in foot and knee surgery (2014-2016), ICBP NS responsible : Dr. I. Titorencu

PN-II-PT-PCCA-2013-4-0816 : ZETTAskin - Rational design and synthesis of smart bioactive scaffolds for the personalized treatment of acute and chronic skin wounds (2014-2016), ICBP NS responsible : Dr. I. Titorencu

PN-II-PT-PCCA-2013-4-958: OsseoPromote - Multifunctional coatings for load bearing implants made of novel titanium-based alloy (2014-2016), ICBP NS responsible: Dr. I. Titorencu

PATENTS

• No A01336/ 7.12.2011, M.G. Albu, I. Lascăr, D. Zamfirescu, M. Simionescu, I. Zegrea, I. Titorencu, M. Popescu, G. Bumbeneci, Nervous conductors made of collagen and process for preparing the same, OSIM Nr. A01336/ 7.12.2011.

• Patent application No 00842/13.07.2017, A. Vlădescu, C. M. Cotruț, A.E. Kiss, M. Braic, I. Titorencu, V. Braic, „Coating magnesium alloys with bioactive layers for medical applications".

• Patent application No. A 00753/27.09.2017, M.G. Albu, I. Lascăr, I. C. Stancu, I. Titorencu, D.G. Zamfirescu, I. Zegrea, S. Marin, A. Lungu, C. Nitipir, R. Țuțuianu, M. Simionescu, "Stratified porous scaffolds for the personalized treatment of difficult wounds and their synthesis process".

AWARDS

Gold Medal for the innovation ***NERVOUS CONDUCTORS MADE OF COLLAGEN AND PROCESS FOR PREPARING THE SAME*** , M. G. Albu, I. Lascăr, D. G. Zamfirescu, M. Simionescu, I. Zegrea, I. Titorencu, M. Popescu, G. Bumbeneci, at the Belgian and International Trade Fair for Technological Innovation, Brussels, 2013.

Prix de l'AGEPI for the invention ***CONDUCTEURS NERVEUX DE COLLAGENE ET LEUR PROCESSUS D'OBTENTION***, M. G. Albu, I. Lascar, D. Zamfirescu, M. Simionescu, I. Titorencu et colab., at the 62nd Edition of the International Salon for Inventions INNOVA, Brussels, 2013.

1st Prize for the invention ***CONDUCTORI NERVOSI DIN COLAGEN SI PROCEDEU DE OBTINERE A ACESTORA***, M. G. Albu, I. Lascar, D. G. Zamfirescu, M. Simionescu, I. Zegrea, I. Titorencu, M. Popescu, G. Bumbeneci, Romanian Innovations Prizes - RADOR, Bucharest, 2014.

3rd Prize for the oral presentation ***MESENCHYMAL STEM CELLS RELEASED FACTORS SUSTAIN THE POPULATION OF COLLAGEN–ALGINATE SCAFFOLDS WITH HUMAN SKIN CELLS FOR DERMAL RECONSTRUCTION,*** R. Țuțuianu, A. M. Rosca, V. Pruna, M. G. Albu Kaya, D. Zamfirescu, I. Titorencu, M. Simionescu, at the PhD Students Symposium, 9th National Congress of the Romanian Society for Cellular Biology, June 2017, Iassy, România.

2nd Prize for the poster ***SILVER-BASED NANOPARTICLES PRESERVE THE PROPERTIES OF KERATINOCYTES AND DERMAL FIBROBLASTS AND CAN BE SAFELY USED FOR SKIN WOUND HEALING***, R. Tutuianu, A. M. Rosca, A. Grumezescu, A. E. Stoica, I. Titorencu, at the 11th National Congress of the Romanian Society for Cellular Biology, June 2019, Constanța, România.

CELL AND TISSUE ENGINEERING LABORATORY