ROMANIAN ACADEMY
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PhD THESIS SUMMARY

EXPLORING THE CAPACITY OF MESENCHYMAL STROMAL CELLS DERIVED SECRETOME COMBINED WITH NANO-BIOMATERIALS TO CONTRIBUTE TOWARDS INNOVATIVE THERAPIES IN REGENERATIVE MEDICINE

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Introduction and general objectives

Regenerative medicine represents a branch of medicine developed in order to improve the patients' quality of life. According to a definition from 2006, this interdisciplinary domain uses different technological approaches such as: administration of soluble molecules, gene therapy, stem cell transplantation, cell reprogramming and mostly, tissue engineering [[Greenwood et al. 2006]. Johnson et al performed an analysis of the type of approaches from this domain, and concluded that it is necessary to understand and control the angiogensis process, together with the immune response of the organism, the behaviour of stem cells and the standardization of their use as well as mass production of the tissue engineering constructs [Johnson et al. 2007]. Therfore, the sinergy between basic sciences sucb as cell biology and material science has led to the development of complex tridimensional (3D) cell-biomaterial structures, which can be implantable with to purpose of regenerating the damaged tissue, as well as systems based exclusively on cells- such as stem cell transplants, or biomaterials, which are capable of integrating within the local tissue after implantation [Muzzio et al. 2021].

One of the main pillars which sustained the development of regenerative medicine, including tissue engineering, was established over 50 years ago, when Friedenstein et al. reported the isolation of a fibroblast-like, clonogenic, multipotent cell population derived from bone marrow [Friedenstein et al. 1968]. Afterwards, these mesenchymal stromal cells (MSC), considered as easy accessible multipotent cells, were also isolated from other human adult sources like adipose tissue or prenatal tissues: umbilical cord, amniotic fluid. MSC were evaluated in clinical studies as cell therapy for different pathologies such as acute myocardial infarction, multiple sclerosis or steroid-resistant acute graft-versus-host disease [NCT00877903, NCT02239393, NCT02923375]. Despite the differential potential revealed *in vitro*, the beneficial effects obtained were not associated with cell grafting or the modification of phenotype towards the one specific for the target tissue. Furthermore, although the preclinical studies have highlighted promising results, the therapeutic effects were not recapitulated in the clinical settings; in this context, the first European market authorization for an MSC based product was just granted in 2018 [Galipeau și Sensebe, 2018]. Meanwhile, the MSC secretome, which comprises the soluble factors (chemokines, cytokines, growth factors) and extracellular vesicles (microvesicles, exosomes) released in the extracellular milieu, has been revealed as the main paracrin effector for the beneficial action observed in the case of administration of these cells, thus outlining a new cell free therapeutic approach [Praveen Kumar et al. 2019].

On the other hand, the material science domain has focused on developing compound with complementary action for MSC. More exactly, considering the extracellular matrix as a model for biomaterials, several tri-dimensional scaffolds have been proposed in order to support the cell proliferation and differentiation needed to promote tissue regeneration. In general, for soft tissue application, polymers such as collagen, alginate, proteoglycans, chitosan have been

employed due to their biocompatibility and bioactivity which allow for cell adherence and growth. At the same time, the nanomaterials domain contributed to the development of regenerative medicine, by providing a variety of nanoparticles which can be used as vectors for active principle release or as antibacterial agents to ensure the safety application of biomaterials, by preventing local infections [Hasan et al. 2018].

In the presented thesis, the following objectives have been proposed:

1. Evaluation of iron based superparamagnetic nanoparticles with polyethylenimine (Fe-PEI NP) obtained via gentle hydrothermal synthesis, which allowed the simultaneous incorporation of therapeutic agents with preserving their action, with the purpose of validating the use of these nanoparticles as vectors for releasing active compounds;
2. Investigation of the capacity of mesenchymal stromal cells-derived secretome to improve the *in vitro* performance of a collagen porous matrix used for skin wound healing treatment;
3. Selection of silver and polymer based nanoparticles with antibacterial effects, based on their biological action on the main cell types involved in skin wound healing: keratinocytes, dermal fibroblasts and endothelial cells;
4. Selection of exosomes secreted by MSC derived from two sources: bone marrow and adipose tissue, in order to validate them *in vitro* as therapeutic agents capable to stimulate skin regeneration via their action on the main phases of skin wound healing

PhD thesis structure

The thesis is structured in two parts: **the first part**- Current state of knowledge- comprises five chapters which present the main characteristics of mesenchymal stromal cells and their therapeutic potential, skin structure and skin wound healing phsases, followed by aspects regarding the applications of nanoparticles, biomaterials and secretome for skin regeneration. The last chapter is dedicated to exosomes, including general aspects concerning their origin, isolation and characterization, focusing on the MSC secreted vesicles as potential therapeutic agents for skin wound healing. **The second part** of the thesis includes the original contributions, organized in four chapters, corresponding to each study employed in order to reach the objectives proposed above.

The first study- *Therapeutic potential of iron based nanoparticles (Fe-PEI NP) as vectors for the controlled release of active compounds* focused on investigating the biocompatibility of superparamagnetic nanoparticles made of iron and poly(ethylenimine) (Fe-PEI NP) on human normal and cancerous cells, while also exploring the capacity of these nanoparticles to incorporate active compounds upon aggregation in cell culture medium followed by their release upon direct contact with the cells. Furthermore, the anti-tumour effect of Fe-PEI NP with cisplatin incorporated during the hydrothermal synthesis was also evaluated on *in vitro* and *in vivo* models.

The main original results are (Fig.1):

1. hybrid nanoparticles with a Fe:PEI mass ratio of 1:2, obtained via hydrothermal synthesis at 100 atm, 40°C and resuspended in NaCl and PAA (polyacrylic acid) solution for stabilization, were associated with a lack of toxicity on mouse mesenchymal stromal cells, with a cytotoxic effect at the same concentrations on glioblastoma cells (U87 cell line);

2. hybrid Fe:PEI nanoparticles aggregated spontaneously in cell culture medium, and the resulted aggregates were able to incorporate active substances (DiI, valinomicyn) present in solution, which they released upon direct cell contact, while preserving the biological activity of the compounds;

3. hybrid Fe-PEI nanoparticles with cisplatin incorporated during synthesis had a stronger anti-tumour effect compared with the drug at same concentration or with the nanoparticles with the drug added after the synthesis, both *in vitro* on the U87 cell line as well as *in vivo* on the mouse model of glioblastoma heterotopic xenograft.

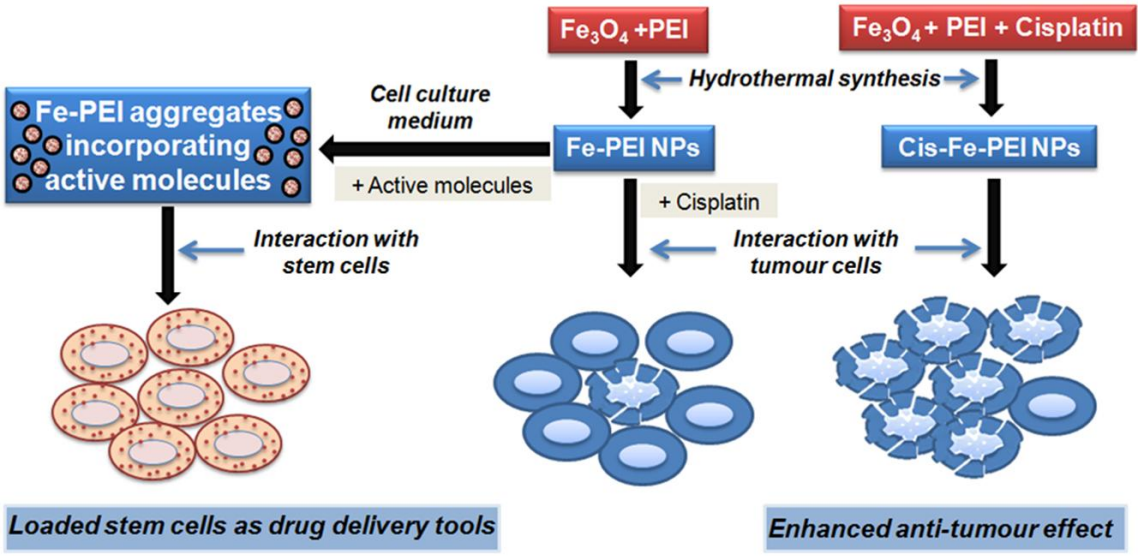

Figure 1. Schematic representation summarising the results presented in the first study concerning the therapeutic potential of hybrid Fe-PEI nanoparticles. The results indicated that NP Fe-PEI can incorporate active molecules by cell culture medium incubation, and the formed aggregates can release the substances upon direct contact with cells. Also, the nanoparticles with cisplatin incorporated during synthesis had a stronger anti-tumour effect compared with the nanoparticles with the drug added after synthesis.

In **the second study**- *Potential of mesenchymal stromal cells-derived secretome for development of skin wound healing therapies*, the improvement of a type I collagen porous

matrix combined with a potent trophic agent: MSC-derived secretome in the form of conditioned medium, was evaluated in terms of the colonization with human keratinocytes and dermal fibroblasts, while also investigation the secretome effect on the *in vitro* proliferation, cell migration and angiogenesis.

The main original results are (Fig.2):

1. MSC secretome promoted the collagen matrix colonization with keratinocytes and fibroblasts, by stimulating their adherence and proliferation;

2. MSC secretome modified the gene expression of molecules involved in synthesis and degradation of extracellular matrix (type I and III collagen, αSMA, TIMP-1, TIMP-2, MMP-9);

3. MSC secretome presented pro-angiogenic properties, exhibiting a motogenic and chemo-attractant effect on endothelial cells.

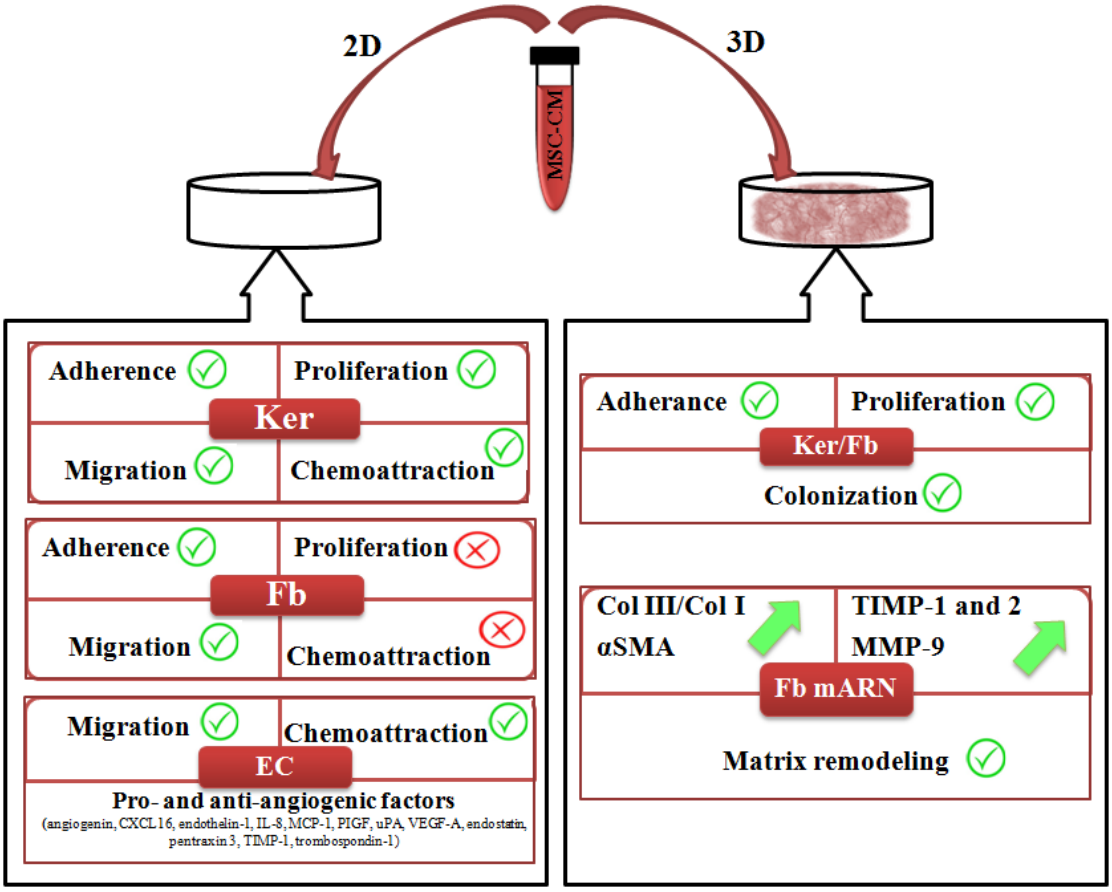

Figure 2. Schematic representation summarising the results presented in the second study concerning the therapeutic potential of the secretome of mesenchymal stromal cells isolated from human bone marrow, combined with a collagen porous matrix indented for the treatment of skin lesions

The third study included in the thesis, addresses the selection of silver based nanoparticles (NP Ag) functionalized with ethylene glycol- EG, poly(ethylene glycol)- PEG with and without poly(vinylpirrolidone)- PVP, to be used as antibacterial agents with the purpose of incorporating then in fibrin based gel for the skin wounds treatment. Choosing the appropriate type of Ag NP was well-founded on a series of experiments, which besides the cytotoxic effect on the viability of keratinocytes, dermal fibroblasts and endothelial cells, also investigated the cell migration and actin cytoskeleton organization, as well as the antibacterial action.

The main original results are (Fig.3):

1. the maximum concentrations for which the cell viability of keratinocytes was not modified compared to control were: 40 µg/ml for Ag EG NP and EG/PVP, 25 µg/ml for Ag PEG NP and 30 µg/ml for Ag PEG PVP NP, respectively

2. the maximum concentrations for which the cell viability of dermal fibroblasts were not modified compared to control, were: 30 µg/ml for Ag EG NP and EG/PVP, 20 µg/ml for Ag PEG NP and 25 µg/ml for Ag PEG PVP NP, respectively

3. the maximum concentrations for which the cell migration was not affected were: 25 µg/ml for Ag EG NP and EG/PVP, and 10 µg/ml for Ag PEG Ag PEG and PVP NP, and the presence of Ag NP induced an increase in the density of actin stress fibers.

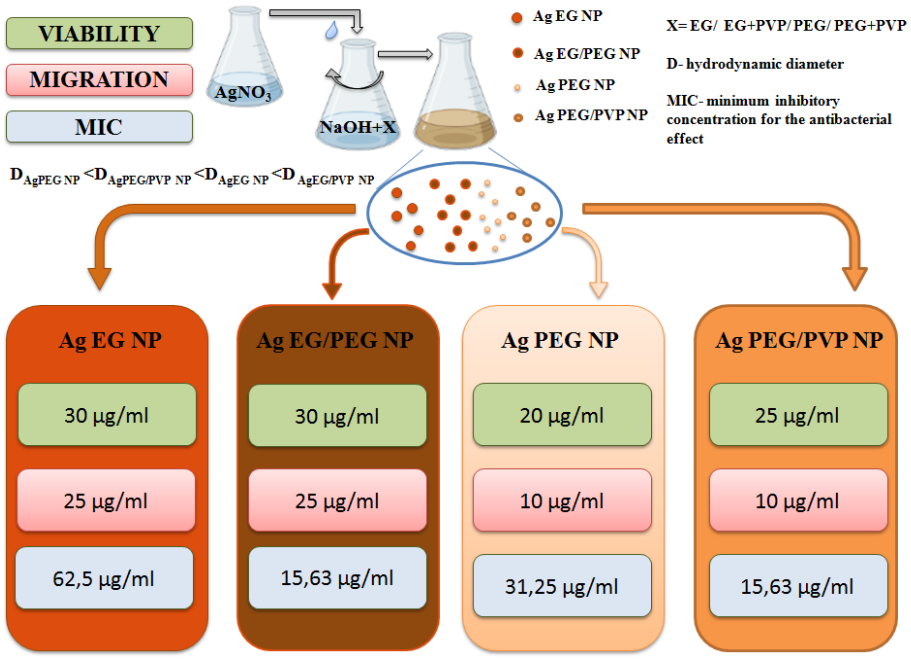

Figure 3. Schematic representation summarising the results presented in the third study regarding the biological activity of silver based nanoparticles obtained via alkaline reduction of silver nitrate,and functionalized with ethylene glycol- EG, polyethylene glycol- PEG, with or without polyvinylpyrrolidone- PVP. From the four types of nanoparticles investigated from

the point of view of cell viability, migratory capacity and minimum inhibitory concentration for the antibacterial effect, the results sustained the use of Ag EG/PVP NP at concentrations up to 25 µg/ml, which did not impact the viability and motility of keratinocytes, dermal fibroblasts and endothelial cells, while also being efficient against the bacteria frequently responsible for skin wounds infections.

The fourth study from the original contributions part, focused on human MSC-derived exosomes, and involved an initial step of selecting the adequate source: MSC isolated from adipose tissue (ADSC) or bone marrow (BM-MSC). The selected exosomes were further investigated concerning their stimulatory action on cell proliferation and migration, angiogenesis, gene and protein expression modifications in the case of fibroblasts differentiated towards myofibroblasts, and the capacity of these nanovesicles to promote the re-epithelialization of a full wound performed on a human skin organotypic model.

The skin organotypic model developed during the PhD training programme, represents an alternative to animal studies, which in the case of skin wounds, lose their relevance for clinical translation because of the major anatomic and physiologic differences compared to humans [Parnell et al 2019]. Furthermore, a similar model is already used for standard skin irritation tests according to ISO 10993-23:2021-Part 23.

The main original results are (Fig. 4):

1. exosomes isolated via differential centrifugation from the bone marrow derived-MSC conditioned medium stimulated the proliferation and migration of human keratinocytes and dermal fibroblasts;

2. upon interaction with dermal fibroblasts, even in a TGFβ1 rich environment, exosomes did not induce a pro-fibrotic phenotype, but rather a pro-healing one, characterized by an increase in the protein expression for type I collagen, fibronectin, decorin and an improvement in contractility, associated with a higher expression of the enzyme lysyl oxidase and not the protein αSMA;

3. exosomes contained factors with pro- and anti-angiogenic action such as Ang-2, ET-1, EG-VEGF/PK1, persefin, uPA and TSP-1, TIMP-1, PEDF, PAI-1, respectively, and these nanovesicles stimulated the endothelial cells migration and tube formation;

4. exosomes sustained the re-epithelialization on a full thickness wounded skin organotypic model, similar to the positive control supplemented with growth factors.

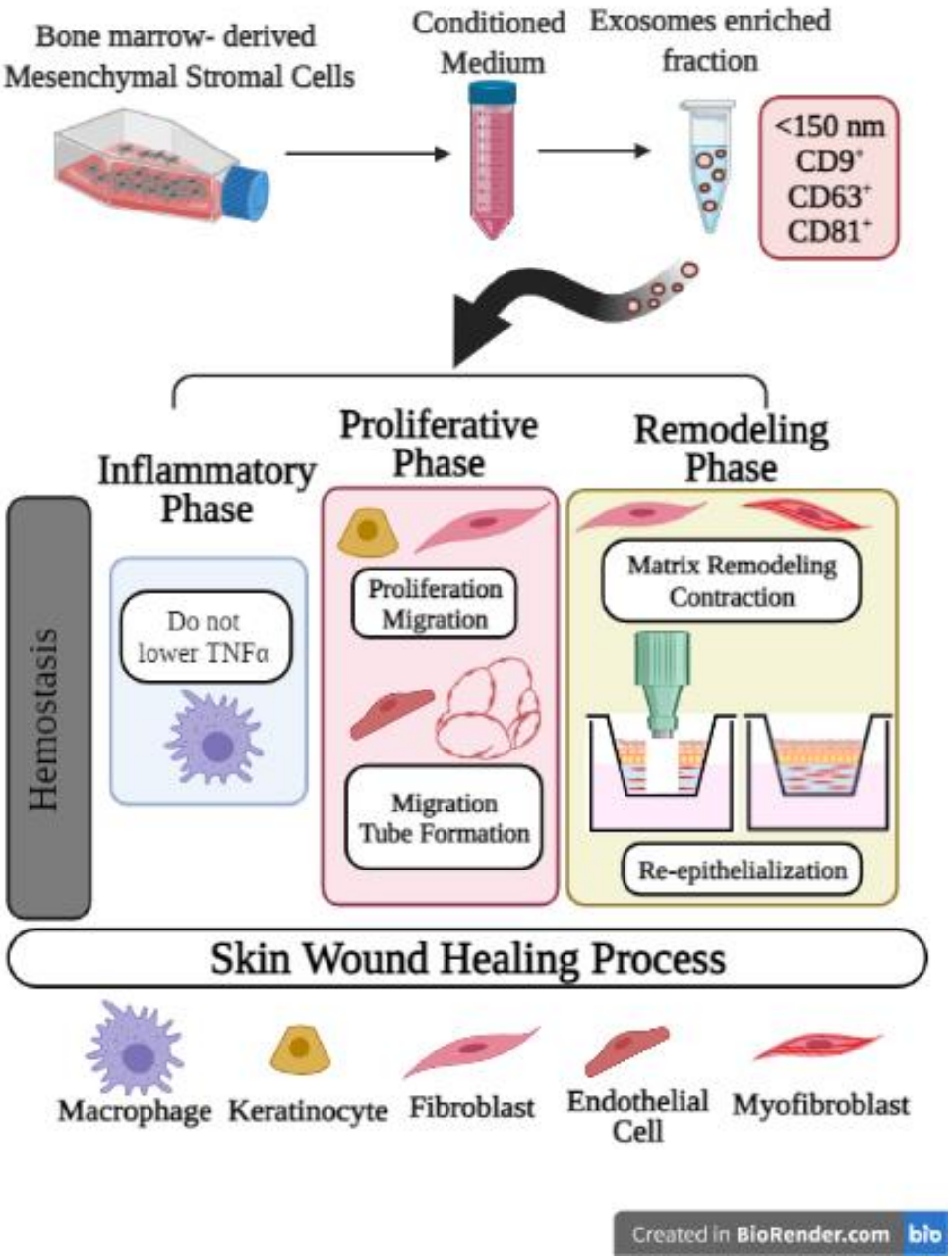

Figure 4. Schematic representation summarizing the results of the fourth study concerning the action of BM-MSC derived exosomes on the main cellular types involved in skin wound healing [created with BioRender.com].

Therefore, from the studies performed during the doctoral training programme, the following original results were obtained:

1. The superparamagnetic iron and poly(ethylenimine) hybrid nanoparticles (Fe-PEI NP) synthesized via the hydrothermal method in mild conditions which supports the incorporation

of active compounds without altering their therapeutic activity, were biocompatibles on both normal and tumour cells. This type of nanoparticles aggregated spontaneously in cell culture medium and allowed the incorporation of active substances in the micrometric resulted structures which were released upon direct cell contact. In the case of incorporating an anti-tumour compound, like cisplatin, during synthesis, at a concentration without secondary effects, the nanoparticles had a cytotoxic effect on glioblastoma cell, and presented a lowering tendency for the size of the tumour *in vivo*, thus supporting the use of Fe-PEI NP as vectors for controlled disease.

2. The secretome, in the form of conditioned medium, derived from human mesenchymal stromal cells (MSC) isolated from bone marrow, had chemo-attractant and mitogen effects on keratinocytes and endothelial cells grown on classical bidimensional culture system. Furthermore, this secretome promoted the colonization with keratinocytes and dermal fibroblasts, of tridimensional porous matrix made of type I bovine collagen. Also, the conditioned medium modulated the gene expression of the molecules involved in the synthesis and degradation of extracellular matrix (type I and III collagen, αSMA, TIMP1, TIMP2, MMP14). Therefore, the resulted data sustained the feasibility of combining the collagen matrix with MSC-derived secretome in the form of an acellular product available "off the shelf" for skin wound healing.

3. The silver based nanoparticles (Ag NP) functionalized with ethylene glycol or poly(ethylene glycol) with and without poly(vinylpirrolidone), synthesized by the silver nitrate reduction method, presented different levels of cytotoxicity for keratinocytes, dermal fibroblasts and endothelial cells; the highest concentration which did not affect the viability and migratory capacity was registered for EG/PVP Ag NP: 25 μg/ml, which, correlated with the minimal inhibitory concentration for the antibacterial effect- 15,63 μg/ml, and thus sustained their use for potential applications in the treatment of skin lesions.

4. The exosomes secreted by MSC isolated from human bone marrow had a greater stimulatory effect on keratinocytes migration compared with the one secreted by MSC isolated from adipose tissue; the selected exosomes improved keratinocytes and dermal fibroblasts, while also inducing a pro-healing phenotype in fibroblasts differentiated towards myofibroblasts, characterized by an increased contractility without the modification of protein expression for αSMA. Furthermore, these nanovesicles sustained endothelial cells migration and tube formation, due to the cytokines and growth factors identified within. Also, the exosomes treatment of the full-thickness lesions performed on human skin organotypic cultures, which mimicked the skin structure, was associated with complete re-epithelialization, thus sustaining the use of this type of extracellular vesicles for skin wound therapy.

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1. **Tutuianu R**, Rosca AM, Iacomi DM, Simionescu M, Titorencu I. Human Mesenchymal Stromal Cell-Derived Exosomes Promote In Vitro Wound Healing by Modulating the Biological Properties of Skin Keratinocytes and Fibroblasts and Stimulating Angiogenesis. Int J Mol Sci. **2021** Jun 9;22(12):6239. doi: 10.3390/ijms22126239. (IF-5,54; Q1).
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2. Rosca AM, Rayia DM, **Tutuianu R**. Emerging Role of Stem Cells - Derived Exosomes as Valuable Tools for Cardiovascular Therapy. Curr Stem Cell Res Ther. 2017;12(2):134-138. doi: 10.2174/1574888X10666151026115320. PMID: 26496883. (IF- 2,16; Q2)

COLABORATOR IN ONE MONOGRAPHY

Rosca AM, **Tutuianu R**, Titorencu I. 2020. Advances in skin regeneration and reconstruction in *Frontiers in Stem Cell and Regenerative Medicine Research*: *vol. 9*, (ed. Atta-ur-Rahman & Shazia Anjum), Bentham Science Publishers, pp. 143-187, ISBN (online): 978-68108-762-7.

PRESENTATIONS AT INTERNATIONAL SCIENTIFIC CONFERENCES

1. Tutuianu R, Rosca AM, Grumezescu AM, Stoica AE, Titorencu I. *In vitro assessment of the effects of silver based nanoparticles on human skin cells involved in the wound healing progress*, 6th Nano Boston Conference, December 7-9 2020, virtual event, e-poster.
2. Tutuianu R, Rosca AM, Grumezescu AM, Stoica AE, Titorencu I. *Silver-based nanoparticles preserve the properties of keratinocytes and dermal fibroblast and can be safely used for skin wound healing,* The 37th Annual Scientific Session of the Romanian Society for Cell Biology and the 11th National Congress with International Participation, June 20-23, 2019, Constanţa, Romania, Book of abstracts p.131 (poster)- second prize at the poster session.
3. Tutuianu R, Rosca AM, Pruna V, Simionescu M, Titorencu I. *The potential of mesenchymal stromal cell secretome for enhancing the skin cells colonization of 3D collagen scaffolds,* The 36th Annual Scientific Session of the Romanian Society for Cell Biology and the 10th National Congress with International Participation, June 6-9, 2018, Craiova, Romania, Book of abstracts p.37 (oral presentation)
4. Tutuianu R, Rosca AM, Pruna V, Albu Kaya MG, Zamfirescu D, Titorencu I, Simionescu M. *Mesenchymal stem cells released factors sustain the population of collagen–alginate scaffolds with human skin cells for dermal reconstruction,* The 35th Annual Scientific Session of the Romanian Society for Cell Biology and the 9th National Congress with International Participation, June 7-11, 2017, Iaşi, Romania, Book of abstracts p. 17 (oral presentation)- third prize at the PhD Students Minisymposium
5. Tutuianu R, Popescu M, Preda MB, Simionescu M, Piticescu R, Bulacu A. *Poly(ethylenimine)-coated iron oxide nanoparticles increase the efficiency of intracellular delivery of biologically active factors incorporated either during or after synthesis*, Conferinţă EMBO "The molecular and cellular basis of regeneration and tissue repair", 17-21 septembrie 2016, Paestum, Italia, Book of abstracts p. 135 (poster)

PATENTS

- Submitted for approval: DSimionescu M, Rosca AM, Titorencu ID, Iacomi MD, Tutuianu R, Pruna V, Lasca I, Chercherita IA, Neagu PT, Mogoanta L, Mogosanu GD, Pirici ND, Streba CT, Birca AC, Burdusel AC, Stoica AE, Grumezescu AL, Chircov C. HIDROGELURI POLIMERICE COMPOZITE CU PROPRIETĂŢI ANTIBACTERIENE ŞI CICATRIZANTE ŞI PROCEDEU DE OBŢINERE A ACESTORA, A/00370 from 25.06.2021

- Approved: Albu MG, Lascar I, Stancu IC, Titorencu I, Zamfirescu DG, Marin S, Lungu A, Nitipir C, Tutuianu R, Simionescu M. SUPORTURI POROASE STRATIFICATE, PENTRU TRATAMENT PERSONALIZAT AL RĂNILOR DIFICILE, ŞI PROCEDEU DE OBŢINERE A ACESTORA, RO-BOPI 3/2019, No. 133133.

SPECIALIZATIONS AND COURSES

1. Training stage on exosome isolation and characterization, under the supervision of Magdalena Lorenowicz, PhD, July 31-August 18, 2018, MSC Biology Group, University Medical Center Utrecht, Netherlands

2. Internation Course "Techniques to validate the isolation of ADSC and their differentiation evaluation", The 2nd CONGRESS of ISRMS (International Society of Regenerative Medicine and Surgery), June 14-16, 2017, Bucharest, Romania.

SCHOLARSHIPS OBTAINED DURING THE DOCTORAL TRAINING PROGRAMME AND FUNDING OD THE RESEARCH ACTIVITY

Doctoral scholarship- Romania Academy (SCOSAAR): 2014-2017

COLLABORATOR IN THE FOLLOWING GRANTS- 6:

1. PROSKIN- Mesenchymal stromal cells contribution to the reepithelialization process of biomimetic skin cultures by differentiation and paracrine factors, PNIII TE 3/2018;
2. NANOLIFE-Bioactive Nanostructures for Innovative Therapeutic Strategies, PN III PCCDI 45/2018;
3. Zettaskin- Rational design and synthesis of bioactive smart scaffolds for the personalized treatment of acute and chronic wounds, PN II PCCA 201/2014
4. Osseopromote- Multifunctional coatings for load bearing implants made of a novel titanium-based alloy, PN II PCCA 212/2014
5. ComTIsM- Ischemic tissue engineering by combinatorial transplant: piecing together the puzzle to gain mutual benefits for graft survival and host tissue repair, PN II TE 83/2014;
6. THERION- Preclinical model of cell therapy employing protein tyrosine phosphatase-microRNA interplay to optimize neovascularization, PN II PCCA 79/2012.