PROGRAMUL DE COOPERARE ELVEȚIANO-ROMÂN
SWISS-ROMANIAN COOPERATION PROGRAMME

Swiss-Romanian Cooperation Programme (SRCP)

Effects of Sex Steroids on Adult Stem/Progentior Cell-Mediated Cardiovascular Regeneration. Grant Agreement between the Romanian and Swiss Beneficiaries, Prof. Dr. Raghvendra K. Dubey (Swiss Beneficiary), Prof. Maya Simionescu (Legal representative of the Romanian Host Institution) (2013-2016).

Swiss-Romanian bilateral cooperation project. Project obtained by Dr. Marilena Plesu (Lupu) as Principal Investigator, and transferred/currently coordinated by Acad. Dr. Maya Simionescu; Funding source: Swiss National Science Foundation/National Authority for Scientific Research.

Objectives: to assess the influence of sex steroids, estradiol and dihydrotestosterone (DHT) on regenerative potential of endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs), using murine cardiac slices; to assess and compare the effects of estradiol and DHT on: (a) the proliferative potential of EPCs and MSCs and (b) the vascular tube-like structures formation by EPCs; to investigate the role of distinct molecular mechanisms in mediating: (a) the proliferation capacity of EPCs and MSCs and (b) the angiogenic/capillary stimulating effects of estradiol and DHT; to investigate the influence of estradiol and DHT on adhesion of EPCs and MSCs to monolayers of arterial endothelium and cardiac fibroblasts, under dynamic (flow) conditions; to screen the modulatory effects of estradiol and DHT on early and late genes associated with tissue regeneration in EPCs and MSCs (angiogenesis / growth / adhesion).

During previous and current work, we have established a novel tissue slice based organ culture system to test the real capability of adult stem cells to repair damaged tissue. Using this system we would like to assess the role of sex hormones in promoting adult stem cell mediated repair of heart tissue.

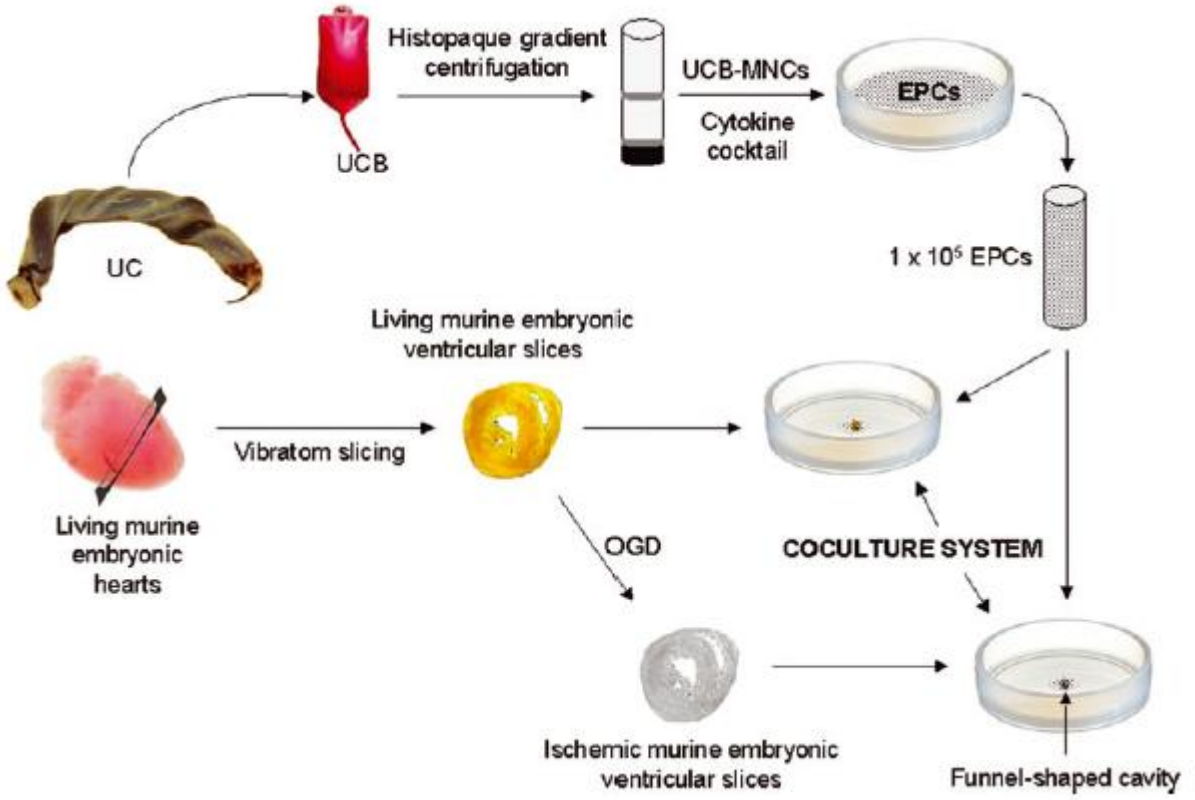

Figure 1. Schematic representation of the experimental design consisting of coculture of EPCs with either living or ischemic murine embryonic ventricular slices. EPCs: endothelial progenitor cells; MNCs: mononuclear cells; OGD: oxygen and glucose deprivation; UC: umbilical cord; UCB: umbilical cord blood.

INTRODUCTION:

Ageing is associated with a significant increase in cardiovascular disease and mortality in both men and women. Because, onset of menopause in women, and andropause in men is associated with a significant decrease in the levels of estrogen and androgens, respectively, it is hypothesized that sex hormones importantly contribute to the increase in cardiovascular mortality in postmenopausal women and men in andropause.
The fact that estrogen potentiates EPCs induced vessel formation in a mouse model for ischemic injury suggests that sex hormones can importantly influence progenitor stem cell-induced tissue repair, however, whether they would mimic similar repair processes in humans remains unknown, moreover, the mechanisms involved remain undefined.

MAIN OBJECTIVE:

Specific Aim #1 (2013-2014): "To assess the influence of sex steroids (estradiol and dihydrotestosterone) on capillary regenerative potential of stem/progenitor cells mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPCs) using human cardiac slices".

We performed the following tasks:

I. Isolation and characterization of EPCs from umbilical cord blood;

II. Transfection of EPCs and MSCs; set up best possible conditions;

III. Set up optimal condition to obtain viable adult murine heart ventricular tissue slices; viability tests;

IV. Testing the effects of estrogens and dihydrotestosterone (DHT) on EPCs and MSCs; identification of hormone receptors; induction of oxidatively stressed EPCs and MSCs (preliminary experiments).

RESULTS OF THE FIRST YEAR:

I. Isolation and characterization of EPCs from umbilical cord blood

To assess the endothelial differentiation, the cultured cells were characterized using an assay based on the ability of differentiated endothelial cells (EC) to form tube-like structures on an extracellular matrix, the Matrigel [1]. It is a quick, easy to set up, and highly reproducible assay to evaluate the vasculogenic capacity of UCB-derived EPCs and HUVECs (as control) and to find out if they form characteristic networks.

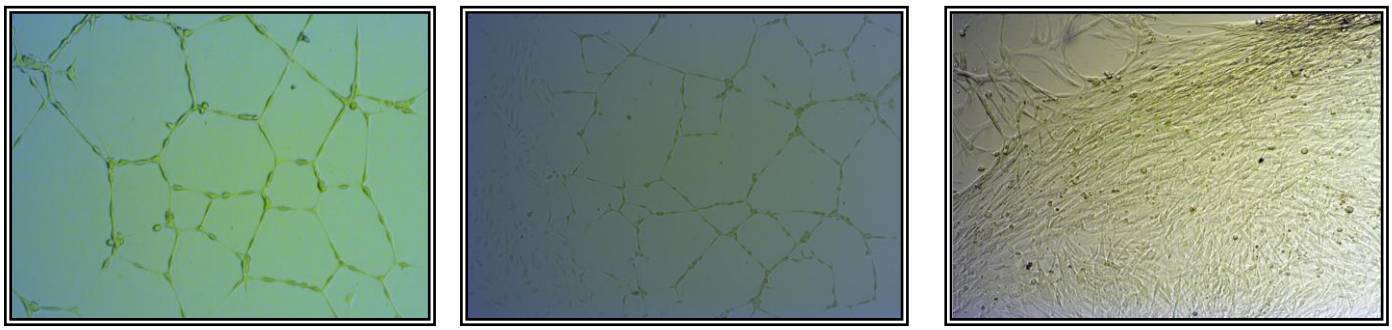

Figure 2. Matrigel Assay performed for (a) EPCs (b) HUVECs, as positive control and (c) MSCs as negative control.
After 24 h in culture, the formation of vascular tube-like structures was assessed employing an inverted microscope equipped with a digital camera system for imaging

Furthermore, the endothelial identity of UCB-derived EPC was confirmed by flow cytometry using the following panel of markers: CD31, CD34, CD44, CD45, CD73, CD90, CD105, CD 144 and CD309. These experiments showed that our three different samples isolated from three patients were positive for CD31, CD44, CD73, CD90, CD105(data not shown).

II. Transfection of EPC and MSC; set up best possible conditions

The co-culture system planned to be used in this project implies the need to employ a combination of cells and adult heart slices. The first step was to mark the cells in order to distinguish them upon incubation with the slices. We had to use a method that will label the cells for a long term and found as appropriate technique the transfection method based on the lipofection protocol.

We obtained an extensive rate of MSC infection with the Plasmidial DNA RFP / GFP after 3-4 weeks of incubation post-antibiotic selection (Figure 3).

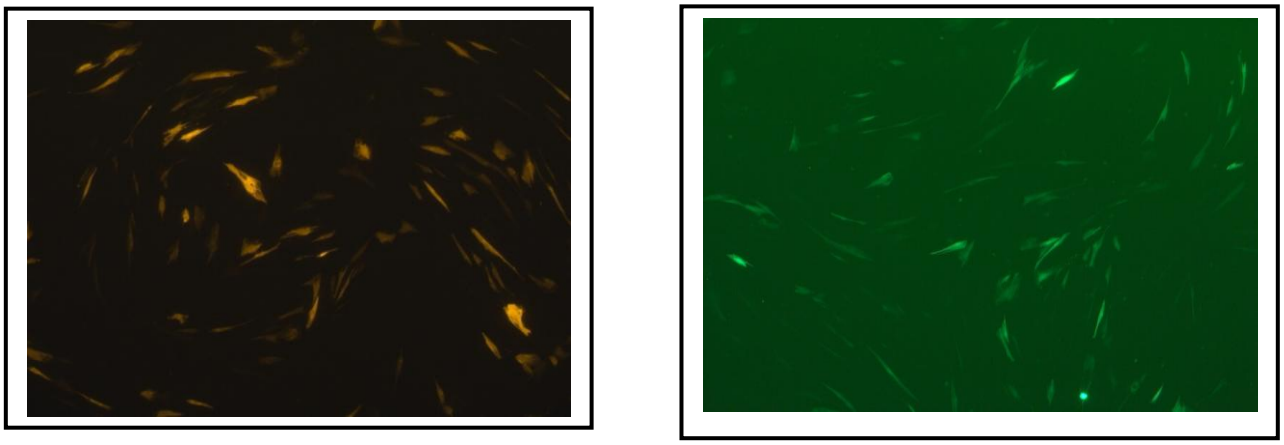

Figure 3. Transfection of MSC with the plasmidial DNA RFP / GFP after 3-4 weeks of incubation post-antibiotic selection

III. Set up optimal condition to obtain viable adult murine heart ventricular tissue slices; viability tests

This part of the project is particularly important and essential for our project because using this system we would like to assess the role of sex hormones in promoting adult stem cell - mediated repair of heart tissue. In recent studies we have demonstrated that human umbilical cord blood-derived EPC had the capacity to integrate and form vascular tube-like structures when in direct contact with living murine embryonic ventricular slices [2]. We tested various thicknesses of the heart sections (150 μm, 200 μm, 250 μm and 300 μm) and the best size chosen to perform further experiments was 250 μm thick (Figure 4).

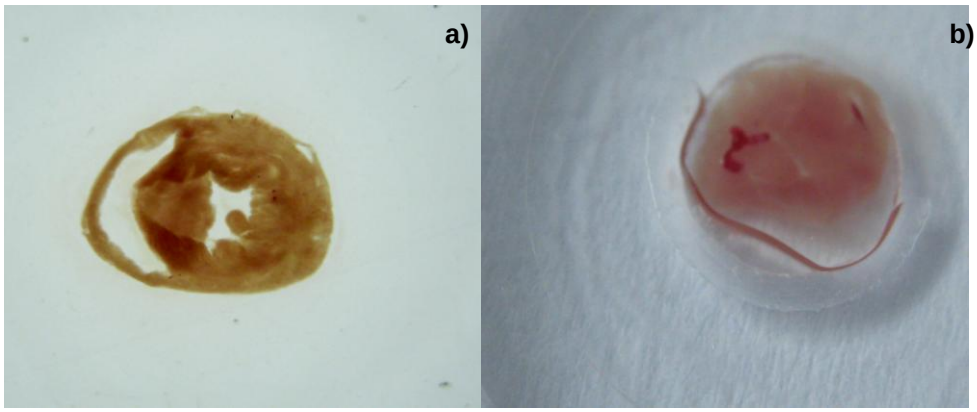

Figure 4. Vibratome sections of adult-heart ventricular tissue slices. a) an adult mouse-heart ventricular slice in culture; b) closer look at the slice included in the co-culture plate

Viability tests :

The murine slices viability were tested by MTT assay and subjected to an analysis of the rapport of the data to dried weight of tissue slices (data not shown) and protein concentration of the slices (Bradford Method). The results confirmed the above results (Figure 5) and we decided for further experiments to employ the slices at maximal 3 days.

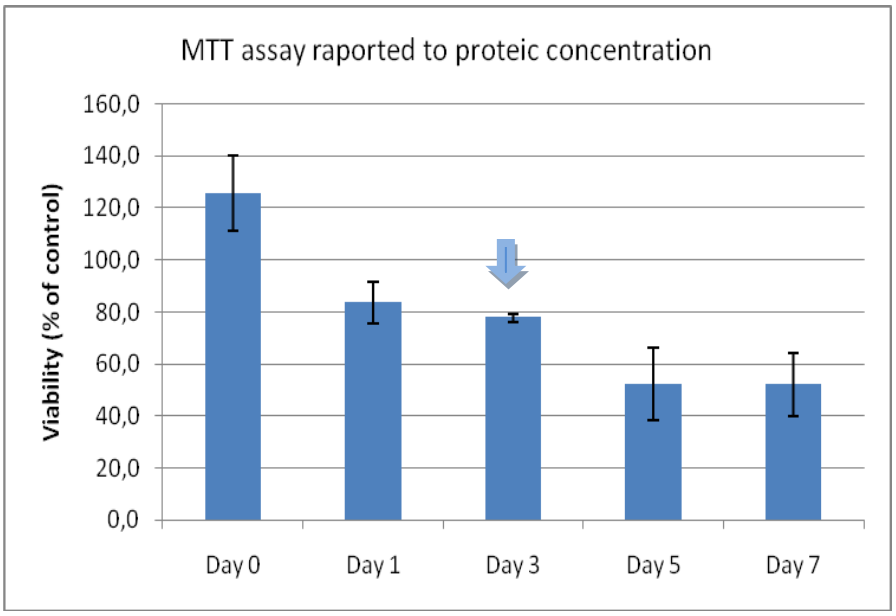

Figure 5. MTT viability assay for murine cardiac slices reported to protein concentration

IV. Testing the effects of estrogens and dihydrotestosterone (DHT) on EPCs and MSCs. Identification of hormone receptors; induction of oxidatively stressed EPCs and MSCs (preliminary experiments).

A) Detection of estrogen receptor (ER) and androgen receptor (AR) on EPCs

It was demonstrated that endothelial cells are significantly involved in estrogen protective activities [3]. Both ERα and ERβ were found in vessels' endothelial cells, with the levels of ERα increasing up to 40-fold after vessel's injury. Recent studies showed the presence of these receptors on stem cells [4].

The results showed that the EPCs population obtained express estrogen and androgen receptor (Figure 6). Similar positive results were obtained on MSC; control studies were consistently negative (data not shown).

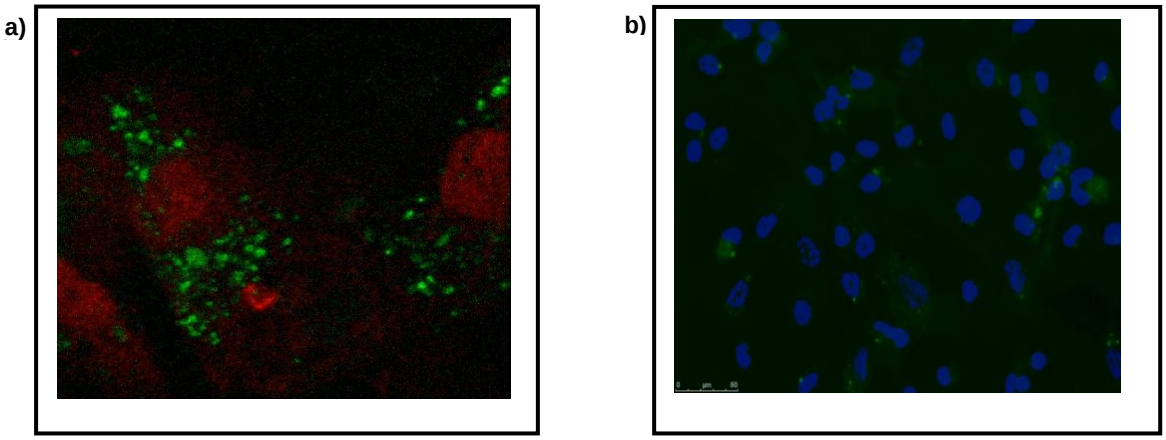

Figure 6. Detection of hormone receptors on EPC by immunofluorescence technique. a) Estrogen Receptor (ER) alpha was localized employing anti-ER alpha antibody [SP1] FITC (green color) and TO-PRO 3 for nuclei staining (red color); b) Androgen Receptor (AR) were detected after incubation with AR Antibody followed by anti-rabbit IgG– FITC produced in goat and staining the cells with DAPI for nuclei (blue color).

B) Determination of the optimal concentration of steroid hormones that induces/maintain proliferation of the EPCs and MSCs

We have done experiments to test the effect of different hormone concentrations on EPCs and MSCs and to assess the increase/decrease proliferation of these stimulated cells and the results revealed that different concentrations of hormones can induce/maintain proliferation of the EPCs and MSCs (data not shown).

Web address of Swiss-Romanian Cooperation Programme (SRPC)

RESULTS DISSEMINATION:

Visit:

Another important activity done this year was the visit of our PhD students, Ms. Corotchi and Mr. Popa to our Swiss partner at the Department of Obstetrics & Gynecology Clinic for Endocrinology (University of Zurich Hospital), Switzerland. During this visit they presented their data to Dr. Dubey's group, had fruitful discussions with the Swiss PI and other members of the department and new plans for future experiments and visits were set up.

Poster presentations at international scientific events :

1**.** „Impact of dihydrotestosterone on human post-natal cord blood and matrix derived adult stem/progenitor cells" , **Popa M.A., Corotchi M.C., Maya Simionescu**; The 32nd Annual Scientific Session of Romanian Society for Cell Biology, 4-7 iunie 2014, Targu-Mures, Romania.

2. „Effects of 17-beta estradiol on proliferation and cardiac integration of human wharton's jelly-derived mesenchymal stem cells" , **Corotchi M.C., Popa M.A., Maya Simionescu**; The 32nd Annual Scientific Session of Romanian Society for Cell Biology, 4-7 iunie 2014, Targu-Mures, Romania.

3. „Impact of dihydrotestosterone on human post-natal cord blood and matrix derived adult stem/progenitor cells" , **Popa M.A., Corotchi M.C., Maya Simionescu**; 7th Santorini Conference Biologie Prospective, 25-27 september 2014; Santorini, Greece.

4. „Effects of 17-beta estradiol on proliferation and cardiac integration of human wharton's jelly-derived mesenchymal stem cells" , **Corotchi M.C., Popa M.A., Maya Simionescu**; 7th Santorini Conference Biologie Prospective, 25-27 september 2014; Santorini, Greece.

References :

1. Crabtree B. Subramanian V. -Behavior of endothelial cells on Matrigel and development of a method for a rapid and reproducible in vitro angiogenesis assay. In Vitro Cell Dev Biol Anim. 2007;43:87.

2. Marilena Lupu , Markus Khalil , Florin Iordache, Eugen Andrei, Kurt Pfannkuche, Dimitry Spitkovsky, Sven Baumgartner, Martin Rubach, Heba AbdelRazik, Cosmin Buzila,Konrad Brockmeier, Maya Simionescu, Jürgen Hescheler , Horia Maniu . Direct contact of umbilical cord blood endothelial progenitors with living cardiac tissue is a requirement for vascular tube-like structures formation J. Cell. Mol. Med. Vol 15, No 9, 2011 pp. 1914-1926.

3. Virginia M Miller, Vesna D Garovic, Kejal Kantarci, Jill N Barnes, Muthuvel Jayachandran, Michelle M Mielke, Michael J Joyner, Lynne T Shuster, Walter A Rocca Sex-specific risk of cardiovascular disease and cognitive decline: pregnancy and menopause. Biol Sex Differ. 2013; 4: 6.

4. Hong SH, et al. Expression of estrogen receptor-alpha and -beta, glucocorticoid receptor, and progesterone receptor genes in human embryonic stem cells and embryoid bodies. Mol Cells.2004;18:320–5.