

Present and future uses of the secretome

for the treatment of cardiovascular,
neurodegenerative, musculoskeletal,
endocrinological and dermatological
conditions

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1. Executive summary

Mesenchymal stem cells (MSCs) are adult stem cells that were originally identified in bone marrow as multi-potent cells. Stem cells are characterized by their self-renewal ability and multi-potency. Adipose-derived MSCs have prominent implications in tissue regeneration due to their high cell yield in adipose tissue, the ability to differentiate into multiple lineages and secrete various cytokines and immunomodulatory effects.

The predominant mechanism by which MSCs participate to tissue repair is through a paracrine activity and exchange of exosomes. Via the production of a multitude of trophic factors with various properties, MSCs can reduce tissue injury, protect tissue from further degradation and/or enhance tissue repair.



The secretome of adipose derived MSC consists of various proteins, micro & messenger RNA and extracellular vesicles, such as, but not limited to, exosomes and microvesicles.

It has been shown that the transplantation of mesenchymal stem cells (MSCs) could be an effective therapy for many diseases including blood disease, acute respiratory distress syndrome, spinal cord injury, liver injury, and critical limb ischemia. To date, hundreds of clinical trials using MSCs have been registered in the database of the US national institutes of health. Furthermore, a number of nonregistered clinical studies using MSCs are being performed in many countries. Throughout these studies safety profile has been very good. No side effects have been recorded during autologous use of stem cells and no major side effects during the use of allogeneic stem cells. The use of cell-free treatments is expected to have an even higher safety profile and lower risk of side effects.

The secretome can be harvested and influenced with various techniques and, although research is still needed for a more precise tailoring of the active substances within the secretome for specific diseases, our patented system (patent deposited worldwide) has proven advantages: high yield of active RNA (messenger and micro RNA) contained in exosomes, microvesicles or free RNA) and cytokines, high amount of therapeutic useful micro RNA such as miR-22, miR-146a, miR-219, low amount of cells necessary for autologous uses, easily scalable production for allogeneic use, standardized production enabling degree of uniformity necessary for identification of active ingredients and thus fulfilling the quality considerations of EMA regarding control and validation of manufacturing steps, purity/consistency and phenotypic/genotypic stability.

Med Cell Europe's system for producing high amount and high quality of active ingredients of the secretomes via EXORAP[®] and EXOPAN[®] will also enable us and our collaborators to develop specific therapies for specific diseases. Our research team has developed a therapy for anti-inflammatory and chondroprotective therapies and is currently working on a specific and focused therapy for cardiovascular diseases, diabetes, Multiple sclerosis, Alzheimer's disease and wound healing.

CM: conditioned medium = secretome

Secretome: extracellular vesicles (exosomes, microvesicles) and cytokines

Paracrine substances = cytokines

miRNA = micro RNA

mRNA= messenger RNA



2. Patented innovation regarding the production of clinical grade secretome

Background

Mesenchymal stem cells (MSCs) are multipotent stromal cells that can differentiate into a variety of cell types, including: osteoblasts, chondrocytes, neurons, muscle cells and adipocytes. This phenomenon has been documented in specific cells and tissues in vivo and in vitro. MSCs are distributed all over the body and are responsible for regeneration. Commonly used tissues for the isolation of MSC are bone marrow, umbilical cord, cord lining and, increasingly, adipose tissue, which has a superior amount of MSCs.

All cells communicate and exchange information by different ways, including the secretion of soluble factors, the cell-to-cell adhesion contact and the intercellular exchange of organelles. The secretome of mesenchymal stem cells includes paracrine substances, exosomes and microvesicles. Over 150 paracrine substances, also called cytokines and chemokines, can be released by mesenchymal stem cells. Two distinct populations of vesicles with peculiar membrane structure, mechanism of production, pathophysiological relevance and different size have been described: exosomes and microvesicles. Microvesicles and exosomes contain biomolecules, including messenger RNA and micro RNA. Exosomes and the whole secretome have been used for therapies in regenerative medicine to treat various illnesses such as osteoarthritis, cardiovascular diseases, neurological diseases, pulmonary diseases, diabetes and many more.

Success rate and duration has been very variable, mainly due to the amount of proteins and RNA used in the studies. We are here presenting our ExoRAP and ExoPAN technology which increases the yield of the secretome by up to 30 times.



Technology

Previous studies suggest that a hypoxic condition promotes self-renewal of undifferentiated mesenchymal stem cells and enhances their therapeutic potential.

Hypoxic exposure activates several signal transduction pathways including hypoxia inducible factor (HIF), a master transcription factor that regulates the expression of hundreds of genes to promote cellular adaptation to the hypoxic condition.

Our ExoRAP and ExoPAN technology uses a protocol of pre-treatment of the cultured MSC in special media and various anoxia conditions instead of hypoxia. With this protocol a 30 fold increase in RNA content per cell can be achieved.

The technology is successfully being used in regenerative treatments in human ExoRAP and ExoPAN technology and animal patients.

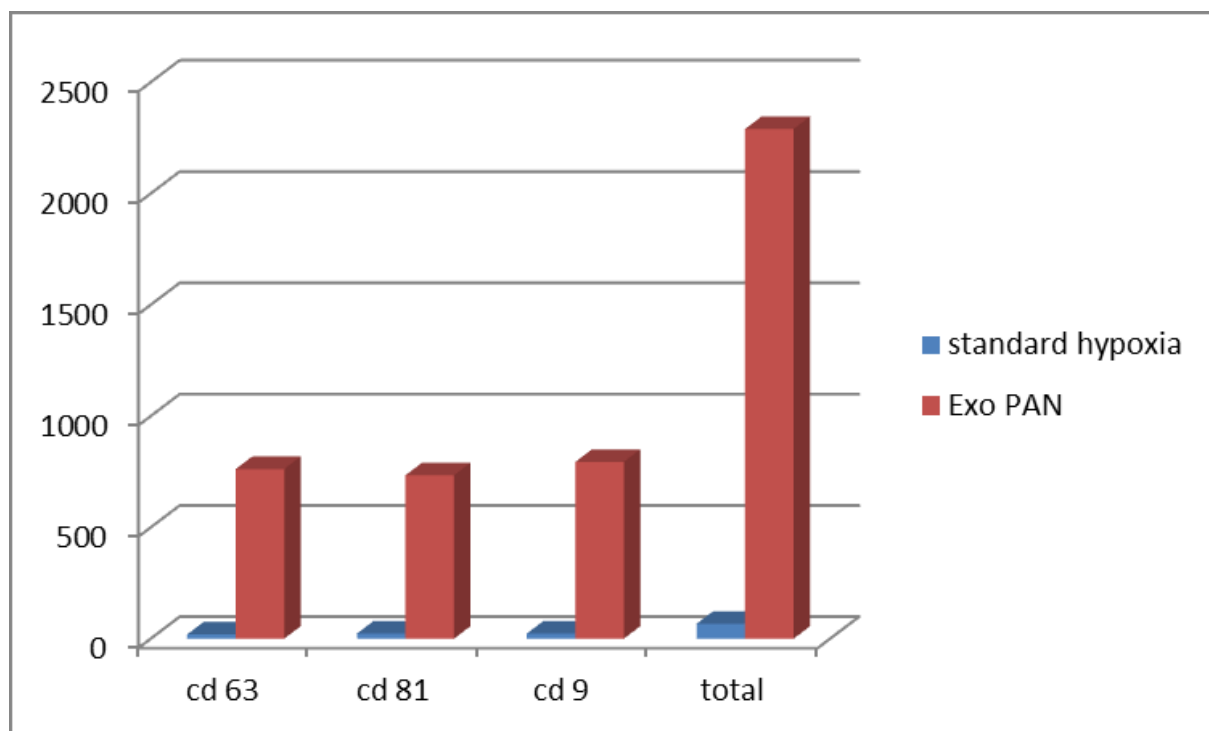


Figure 1: 30 fold increase of exosomes per 1 mio cells as measured semiquantitatively via surface markers cd 63, 81 and 9.

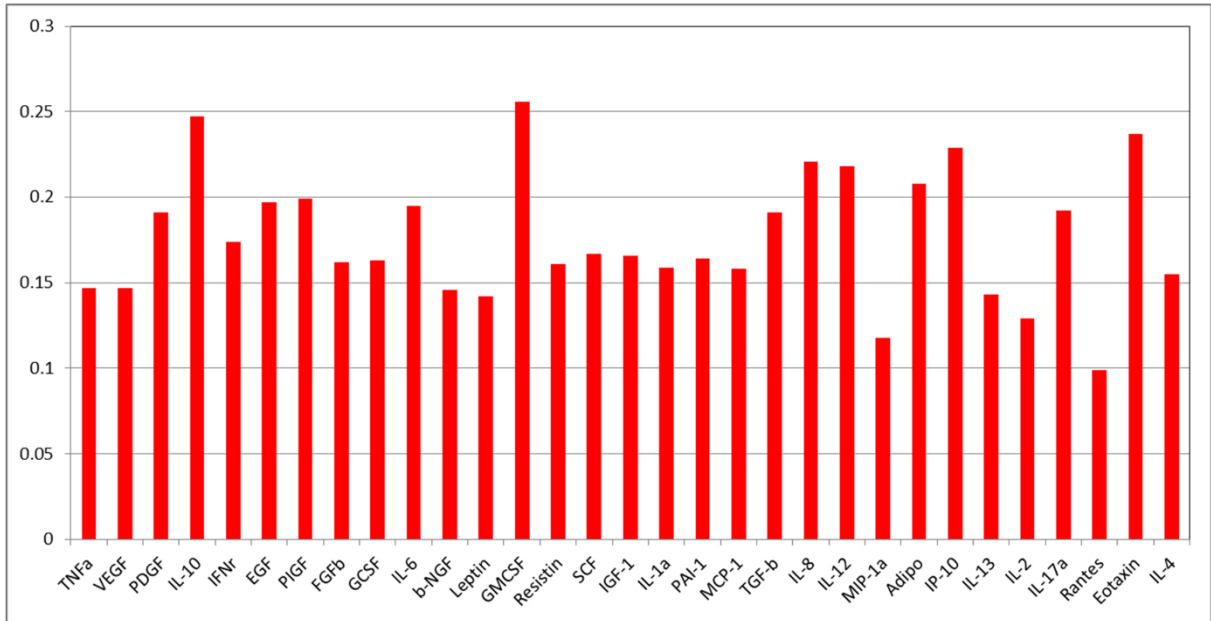


Figure 2: measurement of 31 Cytokines (out of thousands) produced by ad-hMSC

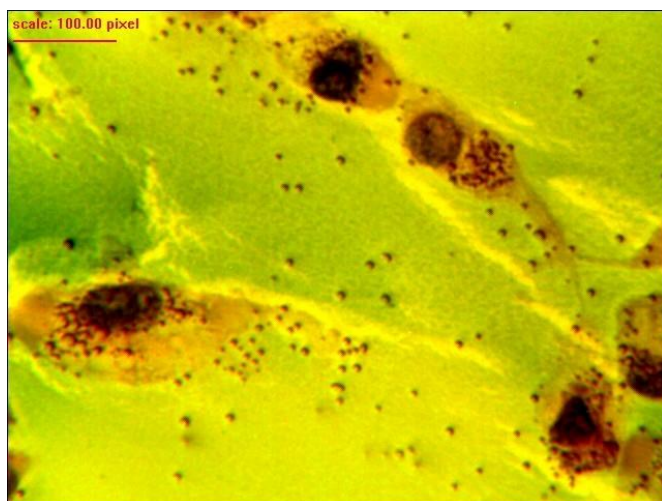
3. Competitive Advantages & Applications

Competitive Advantages

- The EXORAP[®] and EXOPAN[®] technology is easy to use and achieves a much higher amount of RNA. This will be beneficial for all regenerative treatments but especially for diseases where the success rate has not been huge so far, such as diabetes, multiple sclerosis and other neurodegenerative diseases, cardiovascular diseases and further acute or degenerative injuries.
- The secretome is devoid of all cells which will decrease the risk of unwanted reactions in allogeneic use.
- The secretome has been tested in an autologous setting in hundreds of patients with no side effects so far.
- The secretome can be stored at -80°C with minimal loss of activity.
- Therapeutic doses can be achieved with only 1 mio MSCs.

Applications

- Therapies for regenerative medicine for a host of diseases such as osteoarthritis, cardiovascular diseases, lung diseases, liver diseases, neurodegenerative diseases including multiple sclerosis, Parkinson, Alzheimer, GVHD, Crohn's disease and many more.
- Content of specific RNA will enable to focus therapies, such as miR-22 for cardiovascular diseases, miR-133b for neurological diseases, miR-146 for wound healing, miR-204 for pulmonary diseases etc.
- Increase the RNA yield in a research setting, i.e. studies on novel therapies



Literature

The Role of MicroRNA-146a in the Pathogenesis of the Diabetic Wound-Healing Impairment. Junwang Xu et al., *Diabetes* 61:2906–2912, 2012

Hypoxic Conditioned Medium from Human Amniotic Fluid-Derived Mesenchymal Stem Cells Accelerates Skin Wound Healing through TGF- β /SMAD2 and PI3K/Akt Pathways. Eun Kyoung Jun et al., *Int. J. Mol. Sci.* 2014, 15, 605-628

Emerging role of neuronal exosomes in the central nervous system. Mathilde Chivet et al., *frontiers in physiology*, May2012|Volume3|Article145| 3

Exosomal Signaling during Hypoxia Mediates Microvascular Endothelial Cell Migration and Vas- culogenesis. Carlos Salomon et al., www.plosone.org 1 July 2013 | Volume 8 | Issue 7 | e68451

Exosome-Mediated Transfer of miR-133b from Multipotent Mesenchymal Stromal Cells to Neural Cells Contributes to Neurite Outgrowth. Hongqi Xin et al., *Stem Cells*. 2012 July ; 30(7): 1556–1564

Exosomes and microvesicles: extracellular vesicles for genetic information transfer and gene therapy Yi Lee et al., *Human Molecular Genetics*, 2012, Vol. 21, Review Issue 1

Human adipose tissue-derived mesenchymal stem cells secrete functional neprilysin-bound exosomes. Takeshi Katsuda et al., *SCIENTIFIC REPORTS* | 3 : 1197

Exosomes Mediate the Cytoprotective Action of Mesenchymal Stromal Cells on Hypoxia- Induced Pulmonary Hypertension. Changjin Lee et al., *Circulation*. 2012 November 27; 126(22): 2601–2611

Ischemic Preconditioning Potentiates the Protective Effect of Stem Cells through Secretion of Exosomes by Targeting Mecp2 via miR-22. Yuliang Feng et al., *PLOSONE* February 2014 | Volume 9 | Issue 2 | e88685

Transfer of Growth Factor Receptor mRNA Via Exosomes Unravels the Regenerative Effect of Mesenchymal Stem Cells. Susanna Tomasoni et al., *STEM CELLS AND DEVELOPMENT*, Volume 22, Number 5, 2013

New Insight Into Early Events in Type 1 Diabetes: Role for Islet Stem Cell Exosomes. Miodrag L. Lukic et al., *Diabetes* Volume 63, March 2014

4. Licensing opportunities

International Patent (pending) PCT/EP2014/075875, submitted: 27. Nov. 2014

„Secretomes and method for producing secretomes“

Trademarks „EXORAP“, „EXOPAN“ and „EXOSWISS“ filed 6. Nov. 2014

Patent owner currently Med Cell Europe

Patent Inventor: Dr Steven Kellner

Opportunities to partner with Mesencell Biotech International

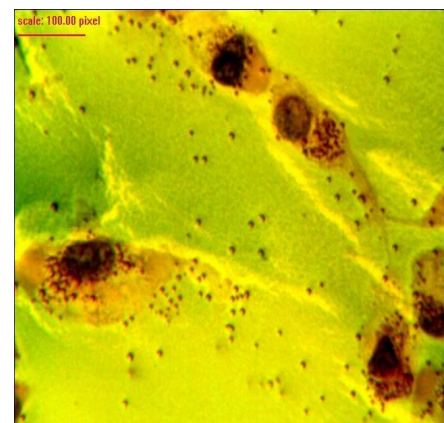
- ☐ Develop therapies in regenerative medicine (osteoarthritis, cardiovascular, lung, liver and neurodegenerative illnesses etc.)
- ☐ In-License our patented know-how, technology and protocols
- ☐ In-vitro studies, R&D on therapeutic exosomes from MSCs & SVFs
- ☐ Know-how exchange on isolation and cryopreservation of MSCs & SVFs and production of cell-free therapies
- ☐ Co-development and R&D on regenerative medicine therapies
- ☐ Therapeutic doses can be achieved with only 1 Mio. MSCs
- ☐ Increase the RNA yield in a research setting, i.e. studies on novel therapies
- ☐ Supply of clinical grade exosomes for studies, R&D
- ☐ R&D for 3rd parties

5. Adipose derived mesenchymal stem cells

Mesenchymal stem cells (MSCs) are adult stem cells that were originally identified in bone marrow as multi-potent cells. Stem cells are characterized by their self-renewal ability and multi-potency. Bone marrow-derived stem cells are most broadly studied for therapeutic potentials since their discovery in the

1960s. After the discovery of bone marrow-derived MSCs, MSCs have been isolated from nearly every tissue in the body, for example, adipose tissue, umbilical cord blood, peripheral blood, dental pulp, dermis, amniotic fluid and even in tumours. Adipose-derived stem cells (ASCs) were first identified as MSCs in adipose tissue in 2001, and since then adipose tissue has been studied as a cell source for tissue engineering and regenerative medicine. There are multiple terms for stem cells derived from adipose tissue, for example, preadipocytes, adipose-derived stromal cells, processed lipoaspirated cells, adipose-derived mesenchymal stem cells, adipose-derived adult stem cells. There are several types of adipose tissue, with subcutaneous as the most clinically relevant source. ASCs can be isolated from subcutaneous adipose tissue of the abdomen, thigh, leg and arm. Because adipose tissue is typically abundant in the human body, ASCs can potentially be isolated in high numbers. The multi-lineage capacity of ASCs offers the potential to repair, maintain or enhance various tissues.

Zuk et al developed a widely used method for isolating ASCs from white adipose tissue in 2001. Adipose tissues are minced and then undergo enzymatic digestion with collagenase type II. After centrifugation, the resulting pellet is called the stromal vascular fraction (SVF). Approximately 2 to 6 million cells in SVF can be obtained from one millilitre of lipoaspirate. SVF contains ASCs, endothelial cells, endothelial progenitor cells, pericytes, smooth muscle cells, leukocytes and erythrocytes. ASCs are obtained as the plastic-adherent population after overnight culturing.



Stem cell yield is higher from adipose tissue than bone marrow-both for aspirated and excised adipose tissues. One gram of aspirated adipose tissue yields approximately 3.5×10^5 to 1×10^6 ASCs. This is compared to 5 hundred to 5×10^4 of bone marrow-derived MSCs (BM-MSCs) isolated from one gram of bone marrow aspirate.

One notable characteristic of ASCs is that they are not a homogenous population[11,26]. Many studies have attempted to characterize ASCs using cell surface markers via flow cytometry analysis, but a unique single marker has yet to be identified. ASCs have a positive expression of CD34 at the first passage of culture, but CD34 expression decreases after passaging. ASCs express typical mesenchymal markers such as CD13, CD29, CD44, CD63, CD73, CD90 and CD105. ASCs are negative for hematopoietic antigens such as CD14, CD31, CD45 and CD144. After culturing and passaging ASC's surface markers can change with passaging. The expression of hematopoietic markers such as CD11, CD14, CD34 and CD45 dissipates or are lost. On the other hand, the expression level of CD29, CD73, CD90 and CD166 increase from the SVF to passage 2. Passaging is considered to select cell population with more homogenous cell surface markers compared to SVF.

ASCs are considered to be a mediator of tissue regeneration through the secretion of specific soluble factors. ASCs secrete multiple growth factors, including basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), insulin-like growth factor 1, hepatocyte growth factors (HGF) and transforming growth factor (TGF)- β 1[32]. The expression of cytokines renders ASCs promising therapies for transplantation and ischemia patients.

ASCs possess unique paracrine characteristics. ASCs secrete growth factors that stimulate recovery of damaged tissue. Furthermore, ASCs express several kinds of growth factor receptors and are sensitive to growth factors. Therefore, ASCs mediate tissue regeneration.

ASCs can be differentiated into multiple lineages under culturing with specific conditions, which results in the potential of ASCs for multiple clinical applications. The induction of ASC differentiation in vitro is achieved by culture with media containing selective lineage-specific induction factors. ASCs have been shown to differentiate into cells of ectodermal, endodermal and mesodermal origin. Less controversial is the differentiation of ASCs into adipogenic, chondrogenic and osteogenic cells, because ASCs are of mesodermal origin.

(Source: Adipose-derived stem cells: Implications in tissue regeneration; Wakako Tsuji, J Peter Rubin, Kacey G Marra; World J Stem Cells 2014 July 26; 6(3): 312-321)

6. Summary of the safety data on adipose tissue derived mesenchymal stem cells

Review of the safety of mesenchymal stem cells for clinical application
(Youwwi Wang et al., Stem Cells International Vol 2012)

Introduction

It has been shown that the transplantation of mesenchymal stem cells (MSCs) could be an effective therapy for many diseases including blood disease, acute respiratory distress syndrome, spinal cord injury, liver injury, and critical limb ischemia. To date, hundreds of clinical trials using MSCs have been registered in the database (<http://www.clinicaltrials.gov/>) of the US national institutes of health. Furthermore, a number of nonregistered clinical studies using MSCs are being performed in many countries.

The general practice includes the isolation of MSCs from various tissues (including bone marrow, adipose tissue, placenta, umbilical cord, umbilical cord blood, peripheral blood and dental pulp) and the cell expansion under in vitro culture conditions. The complications in the utilization of MSCs as therapeutic tools in vivo arose due to the experimental artefacts introduced by in- consistent cell culture protocols. Actually, most MSCs used for clinical trials are prepared in research laboratories, lacking sufficient preclinical studies and manufacturing quality control. Moreover, laboratories around the world lack an internationally standardized practice for in vitro expansion of MSCs, resulting in heterogeneous populations of cells and inconsistent results, both in experimental studies and clinical trials.

In addition, although MSCs have been used in both autologous and allogeneic settings, most clinical applications of MSCs are in fact personalized therapies in which the patient receives administration of MSCs provided by different do- nor and/or different preparation. This necessitates the establishment of standardized manufacture guidelines for the isolation, expansion, preservation, and delivery of MSCs that display minimal variability in their production and assumes large-scale produced MSCs as “cell medicine” for safety evaluation and clinical applications.

Expansion and genetic stability

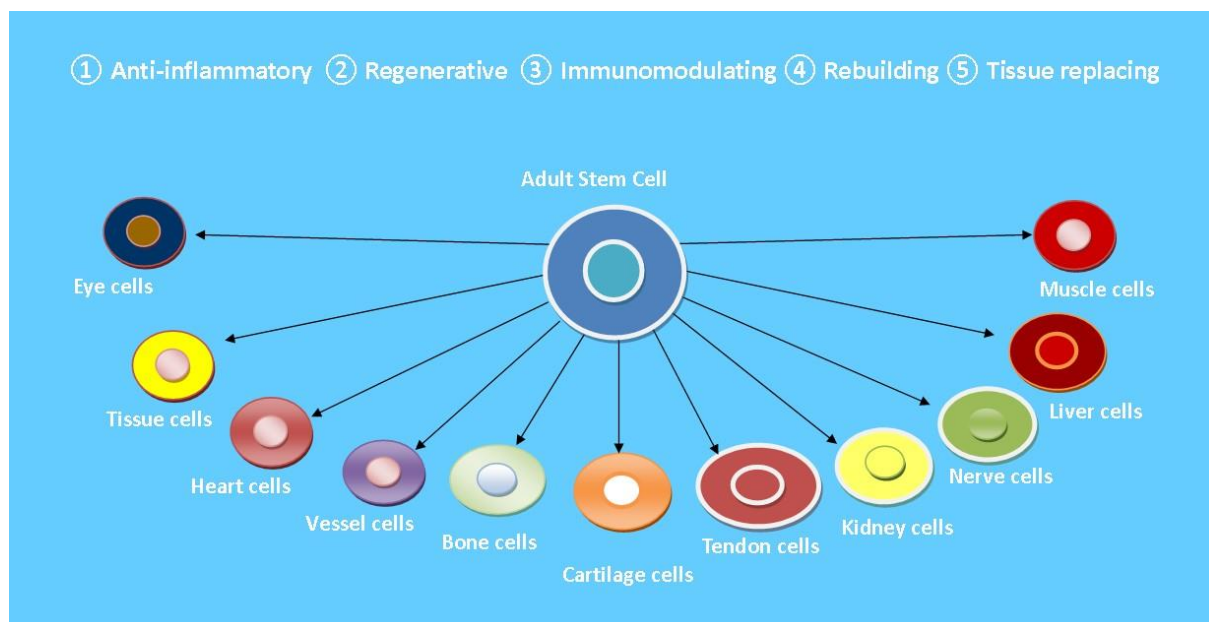
Primary MSCs are rare in human tissues. The frequency of MSCs is approximately 1/106 nucleated cells in adult bone marrow and 1/104 nucleated cells in umbilical cord.

The number of MSCs has been noted to decrease with age. When grouped by decade, a significant decrease in MSCs per nucleated bone marrow cell could be observed, with 10-fold decrease from birth to teens and another 10-fold decrease from teens to elderly.

Despite limited number, MSCs can be expanded to a high level in long-term culture system, which permits a large-scale production of MSCs for clinical application. Usually, the adult bone marrow MSCs (BM-MSCs) can grow identically in culture for 6 –10 passages, whereas placenta umbilical cord MSCs can undergo 30 to 40 passages. It is known that prolonged culture of human embryonic stem cells (ESCs) can lead to adaptation and acquisition of chromosomal abnormalities

The induced pluripotent stem cells (iPSCs) undergo deletions of tumor-suppressor genes during the process of reprogramming, while duplications of oncogenic genes aroused in culture. It remains unclear whether the “culture- adapted” MSCs undergo adaptive transformation during long-term passaging in vitro.

Previous data obtained from high-resolution analysis show that the in vitro expanded human BM-MSCs are devoid of DNA copy number aberrations.



However, senescence-associated modification at specific CpG sites has been observed in MSC during culture expansion. The key evidence for transformation based on DNA fingerprinting has not been presented in the studies in which the authors claimed that MSCs underwent malignant transformation in cultivation. More data are therefore needed to evaluate the genomic stability of MSCs during prolonged culture in vitro. The high resolution of genetic analysis including balanced and unbalanced genetic change is an important method to determine the possibility of transformation of MSCs. If CNV or SNP change is detected after long-term cultivation, it refers not only to the occurrence of mutation but also to the mutation providing a survival (senescence or apoptosis resistance) or growth advantage more or less. Even if there is no difference between early- and late-passage MSCs in genome, it does not imply the absence of genomic alteration during long-term cultivation. The mutated MSCs without survival or growth advantage will be diluted in the process of cultivation and become undetectable after long-term cultivation. By contrast, the mutated MSCs with growth advantage or senescence resistance are of more risk for clinical application.

Tumour formation

Stem cells possess some features of cancer cells including long lifespan, relative apoptosis resistance, and ability to replicate for extended periods of time. In addition, similar growth regulators and control mechanisms are involved in both cancer and stem cell maintenance. Therefore, stem cells may undergo malignant transformation which is often seen as a key obstacle to the safe use of stem-cell-based medicinal products.

Some previous studies have described spontaneous transformation of MSCs in vitro. However, almost all of them have not provided solid evaluation of the same origin of normal MSCs and their transformed counterparts. Actually, most of the spontaneous malignant transformed MSCs are cross-contaminated by HT1080, HELA, or other tumour cell lines.

There is not enough evidence for tumorigenicity of MSCs expanded in vitro.



To address the safety issues, we conducted several GLP-compliant *in vivo* toxicity studies using NOD mice, NOD/SCID mice, guinea pigs, rabbits, and monkey models. UC-MSCs from master MSCs bank (passage 2, P2) were thawed and cultured for additional five passages (P7) and eleven passages (P13). At the end of P7 or P13, an approximate number of 6×10^9 or $5 \times$

10^{12} UC-MSCs were, respectively, harvested, allotted, and cryopreserved until use. For tumourigenic study, UC-MSCs at a dose of 1×10^7 /mouse were subcutaneously transplanted into both NOD mice and NOD/SCID mice. No tumour formation was observed two months after cell transplantation in these animals. The effect of transplanted UC-MSCs on tumour growth was then studied using the NOD mice which were previously injected with K562 cells to induce leukemic tumours. Two injections (two-week interval) of different doses of UC-MSCs resulted in a significant inhibition of K562 tumour growth in the mice bearing leukemic tumours. These *in vivo* results are consistent with our *in vitro* results showing a potent inhibitory effect of UC-MSCs on the proliferation of K562 and HL-60 cells without inducing apoptosis.

In an effort to evaluate the overall toxicology of UC-MSCs, we have performed an *in vivo* study in cynomolgus monkeys receiving repeated administrations of UC-MSC. The administration of UC-MSC was done by intravenous injection once every two weeks for six weeks, with a dose of 2×10^6 or 1×10^7 cells/kg body weight. All animals survived until scheduled euthanasia. No significant MSCs-related changes were found in body weights, clinical signs, haematological/biochemical values, organ weights, or histopathological findings. The results of this toxicity study indicated that the transplantation of UC-MSC did not affect the general health of cynomolgus monkeys.

Moreover, the vast majority of clinical trials conducted with MSCs in regenerative medicine applications have not reported major health concerns. Centeno et al. report that two groups of patients (group 1: $n = 50$; group 2: $n = 290$) between 2006 and 2010 were treated for various orthopaedic conditions with culture-expanded, autologous BM-MSCs. Cells were cultured in monolayer culture flasks using an autologous platelet lysate technique and reinjected into peripheral joints or into intervertebral discs with use of c-arm fluoroscopy. Using both intensive high-field MRI tracking and complications surveillance in

339 patients, no neoplastic complications were detected at any stem cell re-implantation site. MSCs-based therapy has also already been used in other human disease settings, such as graft-versus-host and cardiac disease, with initial reports indicating a good safety profile.

These findings indicate the lack of solid evidence for malignant transformation in vivo following implantation of MSCs for clinical use. Further studies will be required to determine if MSCs can help tumour formation and related mechanisms. Although MSCs with chromosomal alterations did not show any sign of malignant transformation either in vitro or in vivo, it remains uncertain that acquired mutations will induce cellular transformation during the prolonged culture. There exists the possibility that MSCs gain copy number variation during prolonged expansion. Thus, it is necessary to conduct a CGH or SNP array to evaluate the genomic integrity of MSCs before clinical application.

Cryopreservation and Banking

To isolate and produce the MSCs in large scale for clinical use, standardized preparation processes and long-term storage of MSCs isolated from different sources are needed for future clinical applications.

Since HLA-matched adult organ donors are not always available, stem cells derived from several birth-associated perinatal tissues—including cord blood, placenta, and umbilical cord—can be banked as a safeguard against future life-threatening conditions. The clinical grade production and preservation of perinatal MSCs necessitates adhering to cGMP (current Good Manufacturing Practices) to insure the delivery of a “cell drug” that is not only safe but also reproducible and efficient. Cryopreservation of cells permits the transportation of cells between sites, as well as completion of safety and quality control testing. Taken together, banking of MSCs includes the donor determination and sample collection, primary cell isolation and microbiological testing, cell expansion, cryopreservation and banking, and the large-scale cell expansion and preparation of final stem cell products. Strict standards and management are vital to make the stem cell bank work well. Validated SOPs (Standard Operation Procedures) with quality assurance programs are the key factor of a well- designed bank of MSCs. Maintenance of viability, biological characteristics and sterility make the banked MSCs safe and “ready to use.”



It has been demonstrated that cryopreservation does not change the biological behaviour of MSCs such as differentiation, growth and surface marker. Serum and dimethyl sulfoxide (DMSO) are used in research laboratory as cryoprotectant. The major challenge of freezing MSCs is the toxicity of cryoprotectant in clinical use. The toxicity of DMSO is overestimated as it could be weakened by diluting cryopreserved MSCs before clinical use. FBS (fetal bovine serum), BSA (bovine serum albumin), or HSA (human serum albumin) should not be an alternative cryoprotectant for DMSO because of a risk of contamination with human or animal viruses.

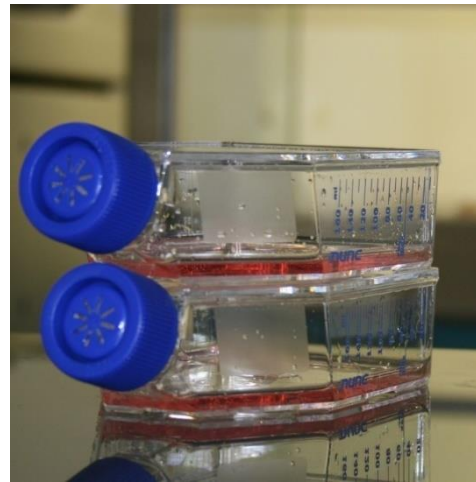
Serum-containing and serum-free cultivations

In vitro expansion of MSCs is conventionally achieved in medium containing FBS and is increased by addition of growth factors. However, for widespread clinical applications, contact of MSCs with serum must be minimized since it is a putative source of prion or virus transmission. Serum is the most uncertain factor in the expansion of clinical grade MSCs. Considering the batch-to-batch variability and possibility of viral contamination, some replacement of FBS such as human serum or platelet lysates cannot be considered as better choice for producing MSCs for clinical use.

Chemical-defined, xeno-free, serum-free medium (SFM) may conquer all the problems of FBS. Furthermore, comparing serum-contained medium, some evidence supported that SFM provided an adjuvant for maintenance of chromosomal stability in BMMSCs and adipose-derived MSCs. Another study focus on mouse embryo fibroblasts showed that predominantly diploid karyotype was maintained in serum-free culture even at PD60. Aneuploid karyotype was induced by the addition of serum. Probably the uncontrolled mitogenic stimulation in serum can lead to strong genetic instability. However, expansion of MSCs in SFM remains an unsolved question. SFM has its own shortcoming in the preparation of clinical grade MSCs. The attachment of MSCs in SFM needs the coating of fibronectin or other substrates which contained components of human origin and batch-to-batch variability and cannot be well defined chemically.

In fact, SFM is not as well as some commercial companies claimed. Based on our own data, UC-MSCs proliferate more slowly in SFM than in FBS-contained media. Sometimes, UC-MSCs cannot be expanded in SFM. A number of studies have revealed that MSCs can be expanded in FBS contained medium without transformation. If MSCs are expanded in SFM, the similar safety studies are needed to determine if the serum-free system affects the genetic stability of MSCs and cause tumour formation.

In summary, the safety remains one of the main concerns in cell therapy. The production of safe cell products requires an entire process supervising to make sure the cells maintain overall phenotype, functional potential and to ensure the cultured cells remain un-transformed and without microbiological contaminations. Therefore, MSCs banking, cell products manufacturing and corresponding quality control system procedures must be applied for assuring the safety and efficiency of the final cell products. Moreover, we cannot only rely on biologists to produce MSCs which fulfils all of the requirements for clinical application. Cell engineering technologies are needed in the translation from expansion of MSCs in laboratory to large-scale manufacture in cell factory.



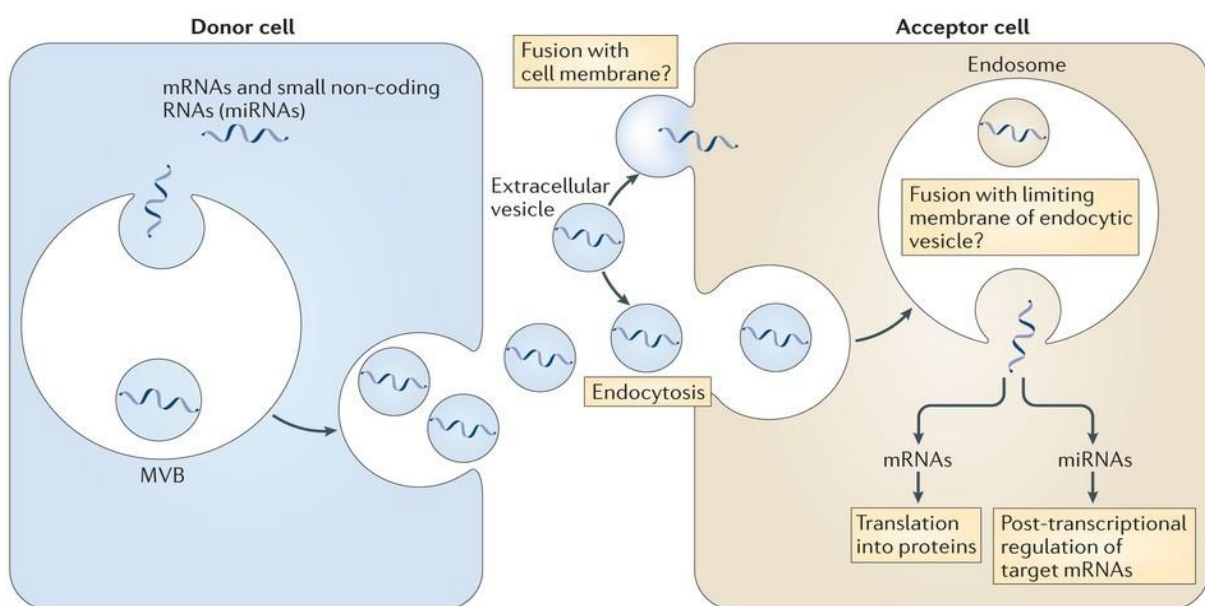
7. The secretome: cytokines and extracellular vesicles

The secretome of adipose derived MSC consists of various proteins, RNA and extracellular vesicles, such as, but not limited to, exosomes and microvesicles.

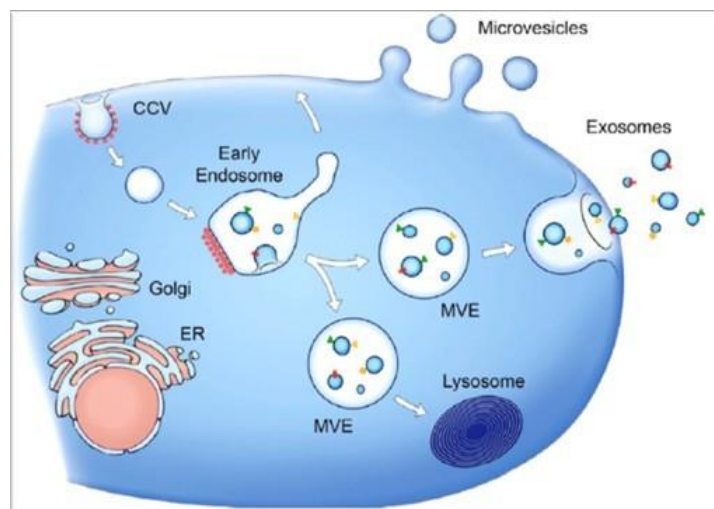
7.1. Introduction to exosomes and microvesicles

Cells communicate and exchange information by different ways, including the secretion of soluble factors, the cell-to-cell adhesion contact and the inter- cellular exchange of organelles through nanotubular structures [1]. Recent studies have proposed that cell-derived small circular membrane vesicles, called exosomes and microvesicles, represent an additional mechanism of cell

-to-cell communication by transfer of membrane components as well as the cytoplasmic content [2,3]. Two distinct populations of vesicles with peculiar membrane structure, mechanism of production, pathophysiological relevance and different size have been described. Membrane fragments of 30 –90nm diameter derived from the endosomal membrane compartment after fusion of secretory granules with the plasma membrane are defined as exosomes. Once released, exosomes bind the recipient cells through receptor – ligand interactions or fuse with the target cell membrane transferring membrane components, including cell receptors [2], and discharging the portion of cytosol segregated within their lumen into the cytoplasm of recipient cells [7]. The molecular cargo content of exosomes derives from active packaging of certain nucleic acid species leading to the presence of mRNAs in exosomes that are not found in donor cells [8].



Furthermore, relatively large microvesicles (100nm–1µm diameter) are formed from the surface membrane of activated cells in a calcium and calpain-dependent manner following a disordered function of phospholipid transporters that results in the budding of altered membrane that exposes phosphatidylserine in the outer leaflet. Microvesicles and exosomes contain biomolecules, including mRNA and microRNA, packaged in a random process and their release is considered an expression of a pathological process in place. Molecular transfer from microvesicles and exosomes contributes to changes in the maturation and differentiation of target cells as for example microvesicles and exosomes released by endothelial progenitor cells trigger neo-angiogenesis in endothelial cells [9].

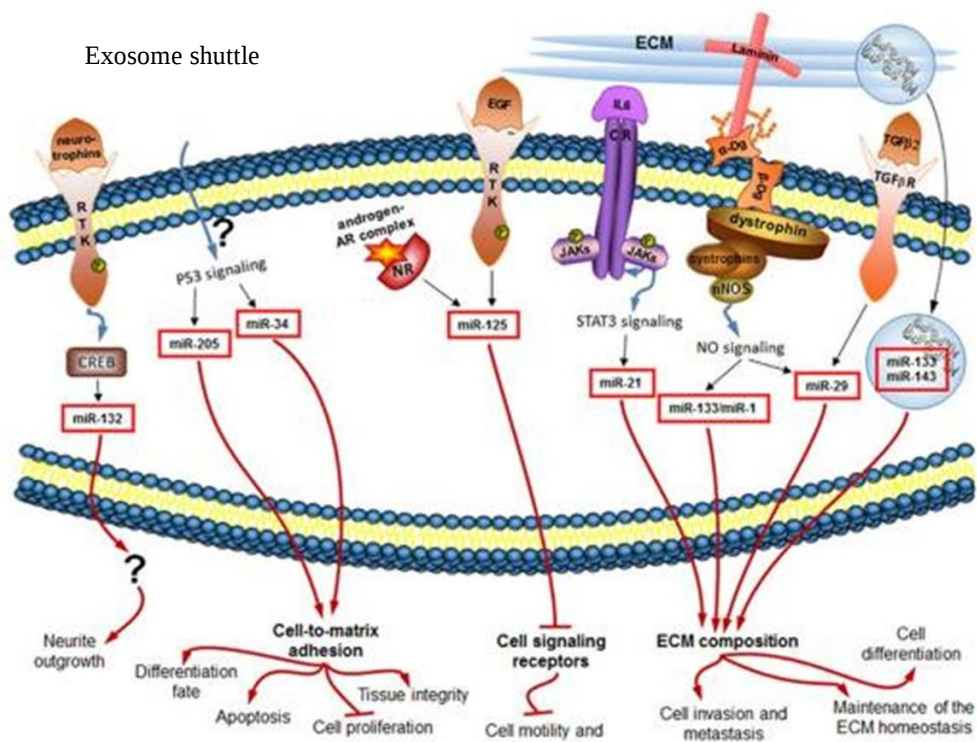


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7.2. Introduction to paracrine substances

MSCs secrete numerous growth factors and cytokines. A typical MSC secretion profile comprises growth factors, cytokines, ECM proteases, hormones, and lipid mediators. Paracrine signaling is a form of cell-to-cell communication in which a cell produces a signal to induce changes in nearby cells, altering the behavior or differentiation of those cells. Signaling molecules known as paracrine factors diffuse over a relatively short distance (local action), as opposed to endocrine factors (hormones which travel considerably longer distances via the circulatory system), juxtacrine interactions and autocrine signaling. Cells that produce paracrine factors secrete them into the immediate extracellular environment. Factors then travel to nearby cells in which the gradient of factor received determines the outcome. However, the exact distance that paracrine factors can travel is not certain. Although paracrine signaling elicits a diverse array of responses in the induced cells, most paracrine factors utilize a relatively streamlined set of receptors and pathways. In fact, different organs in the body -even between different species - are known to utilize a similar sets of paracrine factors in differential development. The highly conserved receptors and pathways can be organized into four major families based on similar structures: Fibroblast growth factor (FGF) family, Hedgehog family, Wnt family, and TGF- β superfamily. Binding of a paracrine factor to its respective receptor initiates signal transduction cascades, eliciting different responses.



8. Studies regarding skin diseases

8.1. The Role of MicroRNA-146a in the Pathogenesis of the Diabetic Wound- Healing Impairment Correction With Mesenchymal Stem Cell Treatment

Junwang Xu, Wenjie Wu, Liping Zhang, Wanda Dorset-Martin, Michael W. Morris, Marc E. Mitchell, and Kenneth W. Liechty

Diabetes 61:2906 –2912, 2012

The impairment in diabetic wound healing represents a significant clinical problem. Chronic inflammation is thought to play a central role in the pathogenesis of this impairment. We have previously shown that treatment of diabetic murine wounds with mesenchymal stem cells (MSCs) can improve healing, but the mechanisms are not completely defined. MicroRNA-146a (miR-146a) has been implicated in regulation of the immune and inflammatory responses. We hypothesized that abnormal miRNA-146a expression may contribute to the chronic inflammation. To test this hypothesis, we examined the expression of miRNA-146a and its target genes in diabetic and nondiabetic mice at baseline and after injury. MiR-146a expression was significantly down-regulated in diabetic mouse wounds. Decreased miR-146a levels also closely correlated with increased gene expression of its proinflammatory target genes. Furthermore, the correction of the diabetic wound healing impairment with MSC treatment was associated with a significant increase in the miR-146a expression level and decreased gene expression of its proinflammatory target genes. These results provide the first evidence that decreased expression of miR-146a in diabetic wounds in response to injury may, in part, be responsible for the abnormal inflammatory response seen in diabetic wounds and may contribute to wound-healing impairment.

It was shown that the cytokine TNF-alpha accelerated wound closure, angiogenesis, proliferation, and infiltration of immune cells into the cutaneous wound in vivo.

8.2. Concise Review: Clinical Translation of Wound Healing Therapies Based on Mesenchymal Stem Cells

WESLEY M. JACKSON, LEON J. NESTI, ROCKY S. TUAN

STEM CELLS TRANSLATIONAL MEDICINE 2012;1:44–50

There is enormous worldwide demand for therapies to promote the efficient resolution of hard-to heal wounds with minimal appearance of scarring. Recent *in vitro* studies with mesenchymal stem cells (MSCs) have identified numerous mechanisms by which these cells can promote the process of wound healing and there is significant interest in the clinical translation of an MSC-based therapy to promote dermal regeneration. This review provides a systematic analysis of recent preclinical and clinical research to evaluate the use of MSCs in wound healing applications. These *in vivo* studies provide overwhelming evidence that MSCs can accelerate wound closure by modulating the inflammatory environment, promoting the formation of a well-vascularized granulation matrix, encouraging the migration of keratinocytes and inhibiting apoptosis of wound healing cells. The trophic effects of MSC therapy also appear to augment wound healing in diabetic tissues, thereby preventing the formation of non-healing ulcers. Finally, a number of delivery systems have been evaluated and indicate that MSCs could be the basis of a versatile therapy to fulfill the clinical needs for dermal regeneration.

Decreased expression of miR-146a in diabetic wounds in response to injury may, in part, be responsible for the abnormal inflammatory response seen in diabetic wounds and may contribute to wound-healing impairment

9. Studies regarding diabetes type 1 and 2

9.1. Research Article: A Modified Method of Insulin Producing Cells' Generation from Bone Marrow-Derived Mesenchymal Stem Cells

Paweł Czubak, Agnieszka Bojarska-Junak, Jacek Tabarkiewicz and Lechosław Putowski

Journal of Diabetes Research Volume 2014, Article ID 628591

Diabetes mellitus is one of major diseases causing heavy burden for many people and countries around the world. These chronic metabolic diseases are caused by absolute (type 1 diabetes) or relative (type 2 diabetes) insulin deficiency leading to hyperglycemia. In both diabetes types, the major determinant for the onset of hyperglycemia and the development of overt disease is an inadequate mass of functional beta cells. Currently, there is no perfect cure for diabetes. Insulin injection does not mimic the precise and dynamic regulation of beta cells on glucose homeostasis, leading on the long term to the development of complications, for example, renal failure or blindness. It also causes a so-called diabetic foot syndrome and results in a tenfold increase in the risk of limb amputation. Many diabetic patients develop hypertension and cardiovascular diseases. Pancreatic islet cells' transplantation is a promising therapeutic option for diabetes mellitus. However, the lack of pancreas donors remains a major obstacle. In recent years, mesenchymal stem cells (MSCs) derived from various tissues have become a research "hot-spot" because of their potential use in regenerative medicine. MSCs are multipotent non hematopoietic progenitor cells. MSCs can be cultured in specially defined conditions and their differentiation extends toward the beta cell phenotype and the

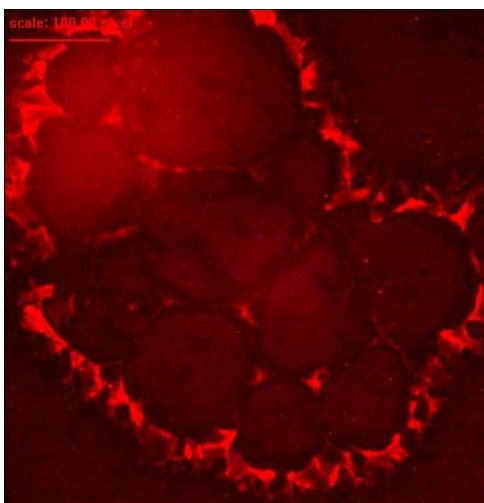
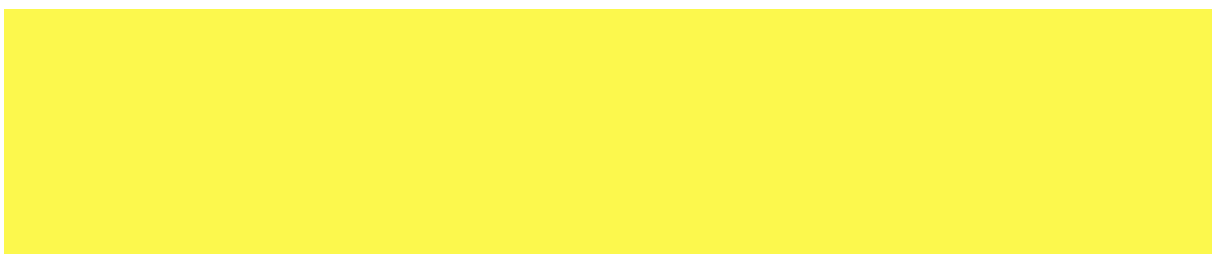
Development towards the beta cell phenotype and the development of insulin producing cells (IPCs).

9.2. Preserved Beta-Cell Function in Type 1 Diabetes by Mesenchymal Stromal Cells.

Carlsson PO, Schwarcz E, Korsgren O, Le Blanc K

Diabetes. 2014 Sep 9. pii: DB_140656. [Epub ahead of print]

The retention of endogenous insulin secretion in type 1 diabetes is an attractive clinical goal, which opens possibilities for long-term restoration of glucose metabolism. Mesenchymal stromal cells (MSCs) constitute, based on animal studies, a promising interventional strategy for the disease. This prospective clinical study describes the translation of this cellular intervention strategy to patients with recent onset type 1 diabetes. Twenty adult patients with newly diagnosed type 1 diabetes were enrolled and randomized to MSC treatment or to the control group. Residual beta-cell function was analyzed as C-peptide concentrations in blood in response to a mixed meal tolerance test (MMTT) at one-year follow-up. In contrast to the patients in the control arm, who showed loss in both C-peptide peak values and C-peptide when calculated as area under the curve during the first year, these responses were preserved or even increased in the MSC-treated patients. Importantly, no side effects of MSC treatment were observed. We conclude that autologous MSC treatment in new onset type 1 diabetes constitute a safe and promising strategy to intervene in disease progression and preserve beta-cell function.



Mesenchymal stem cells from adipose tissue can be transformed into Insulin producing cells in our laboratory.

9.3. Mesenchymal stem cell-based treatment for microvascular and secondary complications of Diabetes mellitus

Grace C. Davey , Swapnil B. Patil ,Aonghus O'Loughlin and Timothy O'Brien

Frontiers in Endocrinology June 2014 | Volume 5 | Article 86

The worldwide increase in the prevalence of Diabetes mellitus (DM) has high- lighted the need for increased research efforts into treatment options for both the disease itself and its associated complications. In recent years, mesenchymal stromal cells (MSCs) have been highlighted as a new emerging regenerative therapy due to their multipotency but also due to their paracrine secretion of angiogenic factors, cytokines and immunomodulatory substances. This review focuses on the potential use of MSCs as a regenerative medicine in microvascular and secondary complications of DM and will discuss the challenges and future prospects of MSCs as a regenerative therapy in this field. MSCs are believed to have an important role in tissue repair. Evidence in recent years has demonstrated that MSCs have potent immunomodulatory functions resulting in active suppression of various components of the host immune response. MSCs may also have glucose lowering properties providing another attractive and unique feature of this therapeutic approach. Through a combination of the above characteristics, MSCs have been shown to exert beneficial effects in pre-clinical models of diabetic complications prompting initial clinical studies in diabetic wound healing and nephropathy. Challenges that remain in the clinical translation of MSC therapy include issues of MSC heterogeneity, optimal mode of cell delivery, homing of these cells to tissues of interest with high efficiency, clinically meaningful engraftment and challenges with cell manufacture. An issue of added importance is whether an autologous or allogeneic approach will be used. In summary, MSC administra- tion has significant potential in the treatment of diabetic microvascular and secondary complications but challenges remain in terms of engraftment, persistence, tissue targeting and cell manufacture.

10. Studies regarding musculoskeletal diseases

10.1. Mesenchymal stem cells in regenerative medicine applied to rheumatic diseases: role of secretome and exosomes.

Maumus M, Jorgensen C, Noël D.

Biochimie. 2013 Dec;95(12):2229-34.

Over the last decades, mesenchymal stem cells (MSCs) have been extensively studied with regard to their potential applications in regenerative medicine. In rheumatic diseases, MSC-based therapy is the subject of great expectations for patients who are refractory to proposed treatments such as rheumatoid arthritis (RA) or display degenerative injuries without possible curative treatment, such as osteoarthritis (OA). The therapeutic potential of MSCs has been demonstrated in several pre-clinical models of OA or RA and both the safety and efficacy of MSC-based therapy is being evaluated in humans. The predominant mechanism by which MSCs participate to tissue repair is through a paracrine activity. Via the production of a multitude of trophic factors with various properties, MSCs can reduce tissue injury, protect tissue from further degradation and/or enhance tissue repair. However, a thorough in vivo examination of MSC-derived secretome and strategies to modulate it are still lacking. The present review discusses the current understanding of the MSC secretome as a therapeutic for treatment of inflammatory or degenerative pathologies focusing on rheumatic diseases. We provide insights on and perspectives for future development of the MSC secretome with respect to the release of extracellular vesicles that would have certain advantages over injection of living MSCs or administration of a single therapeutic factor or a combination of factors.

10.2. Conditioned media from adipose-tissue-derived mesenchymal stem cells downregulate degradative mediators induced by interleukin-1 β in osteoarthritic chondrocytes.

Platas J, Guillén MI, del Caz MD, Gomar F, Mirabet V, Alcaraz MJ

Mediators Inflamm. 2013;2013:357014. doi: 10.1155/2013/357014. Epub 2013 Dec 10.

Osteoarthritis (OA) is the most frequent joint disorder and an important cause of disability. Recent studies have shown the potential of adipose tissue- derived mesenchymal stem cells (AD-MSC) for cartilage repair. We have investigated whether conditioned medium from AD-MSC (CM) may regulate in OA chondrocytes a number of key mediators involved in cartilage degeneration. CM enhanced type II collagen expression in OA chondrocytes while decreasing matrix metalloproteinase (MMP) activity in cell supernatants as well as the levels of MMP-3 and MMP-13 proteins and mRNA in OA chondrocytes stimulated with Interleukin- (IL-) 1 β . In addition, CM increased IL-10 levels and counteracted the stimulating effects of IL-1 β on the production of tumor necrosis factor- α , IL-6, prostaglandin E₂, and NO measured as nitrite and the mRNA expression of these cytokines, CCL-2, CCL-3, CCL-4, CCL-5, CCL-8, CCL-19, CCL-20, CXCL-1, CXCL-2, CXCL-3, CXCL-5, CXCL-8, cyclooxygenase-2, microsomal prostaglandin E synthase-1 and inducible NO synthase. These effects may be dependent on the inhibition of nuclear factor- κ B activation by CM. Our data demonstrate the chondroprotective actions of CM and provide support for further studies of this approach in joint disease.

The MSC secretome with the release of extracellular vesicles has advantages over injection of living MSCs or administration of a single therapeutic factor or a combination of factors

The secretome has a chondroprotective action

10.3. Regenerative Repair of Damaged Meniscus with Autologous Adipose Tissue-Derived Stem Cells

Jaewoo Pak, Jung Hun Lee, and Sang Hee Lee

BioMed Research International Volume 2014, Article ID 436029

Mesenchymal stem cells (MSCs) are defined as pluripotent cells found in numerous human tissues, including bone marrow and adipose tissue. Such MSCs, isolated from bone marrow and adipose tissue, have been shown to differentiate into bone and cartilage, along with other types of tissues. Therefore, MSCs represent a promising new therapy in regenerative medicine. The initial treatment of meniscus tear of the knee is managed conservatively with nonsteroidal anti-inflammatory drugs and physical therapy. When such conservative treatment fails, an arthroscopic resection of the meniscus is necessary. However, the major drawback of the meniscectomy is an early onset of osteoarthritis. Therefore, an effective and noninvasive treatment for patients with continuous knee pain due to damaged meniscus has been sought. Here, we present a review, highlighting the possible regenerative mechanisms of damaged meniscus with MSCs (especially adipose tissue-derived stem cells (ASCs)), along with a case of successful repair of torn meniscus with significant reduction of knee pain by percutaneous injection of autologous ASCs into an adult human knee.

11. Studies regarding neurodegenerative diseases

11.1. IFN γ -stimulated dendritic cell exosomes as a potential therapeutic for remyelination

Aya D. Pusic, Kae M. Pusic, Benjamin L.L. Clayton, Richard P. Kraig

Journal of Neuroimmunology 266 (2014) 12 –23

Dendritic cells (DCs) release exosomes with different characteristics based on stimulus. Here, we showed that DC cultures stimulated with low-level IFN γ released exosomes (IFN γ -DC-Exos) that contained microRNA species that can increase baseline myelination, reduce oxidative stress, and improve remyelination following acute lysolecithin-induced demyelination. Furthermore, nasally administered IFN γ -DC-Exos increased CNS myelination in vivo. IFN γ -DC-Exos were preferentially taken up by oligodendrocytes, suggesting that they directly impact oligodendrocytes to increase myelination. Thus, our results show great potential for use of these IFN γ -DC-Exos as a therapeutic to promote remyelination in multiple sclerosis and dysmyelinating syndromes.

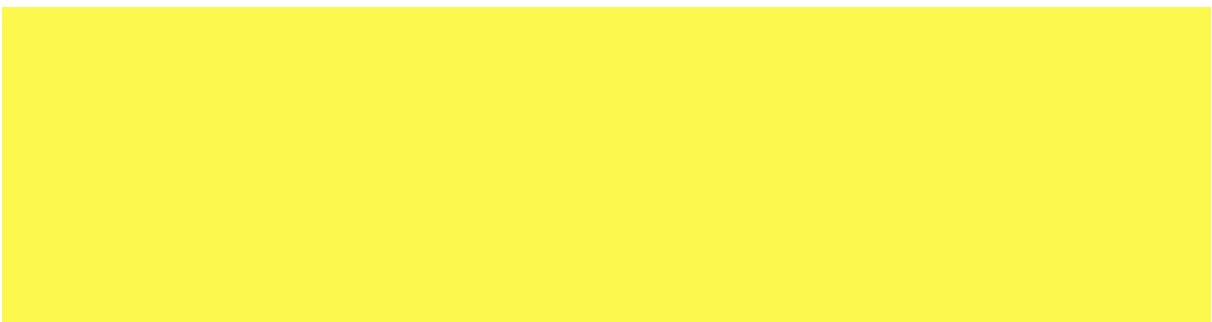
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11.2. Human adipose tissue-derived mesenchymal stem cells secrete functional neprilysin-bound exosomes

Takeshi Katsuda, Reiko Tsuchiya, Nobuyoshi Kosaka, Yusuke Yoshioka, Ken-taro Takagaki, Katsuyuki Oki, Fumitaka Takeshita, Yasuyuki Sakai, Masahiko Kuroda & Takahiro Ochiya

SCIENTIFIC REPORTS | 3 : 1197

Alzheimer's disease (AD) is characterized by the accumulation of b-amyloid peptide (Ab) in the brain because of an imbalance between Ab production and clearance. Neprilysin (NEP) is the most important Ab-degrading enzyme in the brain. Thus, researchers have explored virus-mediated NEP gene delivery. However, such strategies may entail unexpected risks and thus exploration of a new possibility for NEP delivery is also required. Here, we show that human adipose tissue-derived mesenchymal stem cells (ADSCs) secrete exosomes carrying enzymatically active NEP. The NEP-specific activity level of 1 mg protein from ADSC-derived exosomes was equivalent to that of 0.3 ng of recombinant human NEP. Of note, ADSC-derived exosomes were transferred into N2a cells, and were suggested to decrease both secreted and intracellular Ab levels in the N2a cells. Importantly, these characteristics were more pronounced in ADSCs than bone marrow-derived mesenchymal stem cells, suggesting the therapeutic relevance of ADSC-derived exosomes for AD.



11.3. Systemic administration of exosomes released from mesenchymal stromal cells promote functional recovery and neurovascular plasticity after stroke in rats

Hongqi Xin¹, Yi Li, Yisheng Cui, James J Yang, Zheng Gang Zhang and Michael Chopp

Journal of Cerebral Blood Flow & Metabolism (2013) 33, 1711 – 1715

Here, for the first time, we test a novel hypothesis that systemic treatment of stroke with exosomes derived from multipotent mesenchymal stromal cells (MSCs) promote neurovascular remodeling and functional recovery after stroke in rats. Adult male Wistar rats were subjected to 2 hours of middle cerebral artery occlusion (MCAo) followed by tail vein injection of 100 mg protein from MSC exosome precipitates or an equal volume of vehicle phosphate-buffered saline (PBS) (n=6/group) 24 hours later. Animals were killed at 28 days after stroke and histopathology and immunohistochemistry were employed to identify neurite remodeling, neurogenesis and angiogenesis. Systemic administration of MSC-generated exosomes significantly improved functional recovery in stroke rats compared with PBS-treated controls. Axonal density and synaptophysin-positive areas were significantly increased along the ischemic boundary zone of the cortex and striatum in MCAo rats treated with exosomes compared with PBS control. Exosome treatment significantly increased the number of newly formed doublecortin (a marker of neuroblasts) and von Willebrand factor (a marker of endothelial cells) cells. Our results suggest that intravenous administration of cell-free MSC-generated exosomes post stroke improves functional recovery and enhances neurite remodeling, neurogenesis, and angiogenesis and represents a novel treatment for stroke

Systemic administration of cell-free MSC-generated exosomes post stroke improves functional recovery and enhances neurite remodeling, neurogenesis, and angiogenesis and represents a novel treatment for stroke

12. Studies regarding cardiovascular diseases

12.1. Ischemic Preconditioning Potentiates the Protective Effect of Stem Cells through Secretion of Exosomes by Targeting Mecp2 via miR-22

Yuliang Feng, Wei Huang, Mashhood Wani, Xiyong Yu, Muhammad Ashraf

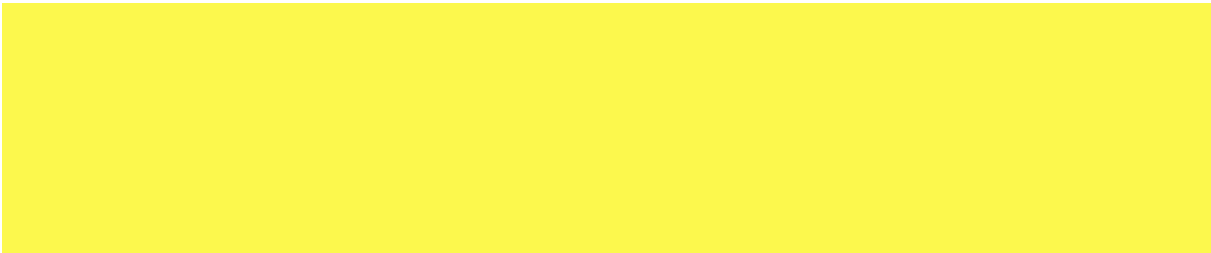
Mesenchymal stem cells (MSCs) have potential application for the treatment of ischemic heart diseases. Besides differentiation properties, MSCs protect ischemic cardiomyocytes by secretion of paracrine factors. In this study, we found exosomes enriched with miR-22 were secreted by MSCs following ischemic preconditioning (ExoIPC) and mobilized to cardiomyocytes where they reduced their apoptosis due to ischemia. Interestingly, by time-lapse imaging, we for the first time captured the dynamic shedding of miR-22 loaded exosomes from cytosol to extracellular space. Furthermore, the antiapoptotic effect of miR-22 was mediated by direct targeting of methyl CpG binding protein 2 (Mecp2). In vivo data showed that delivery of ExoIPC significantly reduced cardiac fibrosis. Our data identified a significant benefit of ExoIPC for the treatment of cardiac diseases by targeting Mecp2 via miR-22.

12.2. Exosomes as Critical Agents of Cardiac Regeneration Triggered by Cell Therapy

Ahmed Gamal-Eldin Ibrahim, Ke Cheng, and Eduardo Marbá'n

Stem Cell Reports j Vol. 2 j 606 –619 j May 6, 2014

The CADUCEUS trial of cardiosphere-derived cells (CDCs) has shown that it may be possible to regenerate injured heart muscle previously thought to be permanently scarred. The mechanisms of benefit are known to be indirect, but the mediators have yet to be identified. Here we pinpoint exosomes secreted by human CDCs as critical agents of regeneration and cardioprotection. CDC exosomes inhibit apoptosis and promote proliferation of cardiomyocytes, while enhancing angiogenesis. Injection of exosomes into injured mouse hearts re-capitulates the regenerative and functional effects produced by CDC trans-plantation, whereas inhibition of exosome production by CDCs blocks those benefits. CDC exosomes contain a distinctive complement of microRNAs, with particular enrichment of miR-146a. Selective administration of a miR-146a mimic reproduces some (but not all) of the benefits of CDC exosomes. The findings identify exosomes as key mediators of CDC-induced regeneration, while highlighting the potential utility of exosomes as cell-free therapeutic candidates.



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*Leaders in isolation, banking & production of
secretomes from adipose derived adult stem cells*



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