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USE OF GENES AND CELLS IN REGENERATIVE MEDICINE

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Use of Genes and Cells in Regenerative medicine

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family

ABSTRACT

Regenerative medicine is a discipline that aims to achieve regeneration of cells, tissue or organs in order to restore or establish normal functions. There are several strategies that can be used to achieve this goal. Many of the strategies are based on use of genes or cells to regenerate organ functions. The present thesis aim to investigate different gene and cell based methods for the use in regenerative medicine.

In paper I a novel peptide conjugate is described for the use of gene transfer *in vitro* and *in vivo*. It shows that serum-resistant nanoparticles are formed upon co-incubation of Stearyl-TP10 with plasmid DNA and that these nanoparticles efficiently transfect cells ubiquitously with minimal toxicity. It is also shown that stearyl-TP10/plasmid nanoparticles enable efficient dose-dependent gene delivery *in vivo* when being administered intramuscularly or intradermally without any associated observed toxicity or induction of immune response. In vivo transfection was highly dependent on a specific charge ratio; this stands in contrast to the in vitro response. Altogether these results show that stearyl-TP10 is an attractive non viral, peptide-based mediator of plasmid delivery, that is effective both *in vitro* and *in vivo*. This peptide based vector could be adapted for delivery of other nucleic acids.

Paper II investigates natural conditions that allow clonal survival and self-renewal of human embryonic stem (ES) cells. Only two laminins, LN-511 and LN-521, could support long-term self-renewal of the cells in a completely defined and xeno-free environment but, unlike LN-511, LN 521 permits survival of individualized human ES or induced pluripotent stem (iPS) cells plated at certain densities.

The paper also shows that the use of LN-521 and E-cadherin together as a culture matrix supports both derivation and clonal survival of hES cells. The paper demonstrates that a single cell obtained by biopsy from an 8-cell human *in vitro* fertilized (IVF) embryo can give rise to new human ES cell lines under completely chemically defined and xeno-free conditions. The methods developed here may have significance for research and clinical applications of human ES cells or other cell types.

Paper III describes the treatment with messenchymal stromal cells (MSC) in two patients with severe refractory ARDS on extracorporeal membrane oxygenation. (ECMO) Although no conclusions could be drawn on effectiveness of MSC treatment in ARDS, clinical improvement was shown after MSC treatment allowing discontinuation of ECMO support and, subsequently, a progressive decrease in the need for mechanical ventilation . Apart from monitoring the clinical progress the *in vivo* actions of the MSCs on lung and systemic inflammation are correlated to vitro potency assays, including effects on inflammatory and immune modulatory cells. Also proteomic assessments of the MSCs and extra cellular vesicles (EV) released by the MSCs are described. This paper outlines a case report and is now planned to be expanded in to a phase 1 clinical trial with MSC treatment to ARDS patients ECMO support.

LIST OF SCIENTIFIC PAPERS

I. Lehto T*, **Simonson OE***, Mäger I, Ezzat K, Sork H, Copolovici DM, Viola JR, Zaghloul EM, Lundin P, Moreno PM, Mäe M, Oskolkov N, Suhorutšenko J, Smith CIE, Andaloussi SE. A peptide-based vector for efficient gene transfer *in vitro* and *in vivo Molecular Therapy* 2011 Aug;19(8):1457-67
L.T., and S.OE. contributed equally

II. Rodin S, Antonsson L, Niaudet C, **Simonson OE**, Salmela E, Hansson EM, Domogatskaya A, Xiao Z, Damdimopoulou P, Sheikhi M, Inzunza J, Nilsson AS, Baker D, Kuiper R, Sun Y, Blennow E, Nordenskjöld M, Grinnemo KH, Kere J, Betsholtz C, Hovatta O, Tryggvason. Clonal culturing of human embryonic stem cells on laminin-521/E-cadherin matrix in defined and xeno-free environment *Nature Communications* 2014;5:3195

III. **Simonson OE**, Mougiakakos D, Heldring N, Bassi G, Johansson HJ, Dalén M, Jitschin R, Rodin S, Corbascio M, El Andaloussi S, Wiklander OP, Nordin JZ, Skog J, Romain C, Koestler T, Hellgren-Johansson L, Schiller P, Joachimsson PO, Hägglund H, Mattsson M, Lehtiö J, Faridani OR, Sandberg R, Korsgren O, Krampera M, Weiss DJ, Grinnemo KH, Le Blanc K. *In vivo* effects of mesenchymal stromal cells in two patients with severe acute respiratory distress syndrome. *Stem Cells Translational Medicine*. 2015 Oct;4(10):1199-213.
S.OE, M.D, W. DJ, G. KH, L.K contributed equally

CONTENTS

LIST OF ABBREVIATIONS

ES cells	Embryonic stem cells
iPSC	Induced pluripotent stem cells
SCID	Severe combined immune deficiency
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
CPP	Cell penetrating peptide
MSC	Messenchymal Stromal Cells
HLA-G	Human leukocyte antigen-G
ECM	Extracellular matrix
LN	Laminin
miRNA, miR	Micro RNA
EV	Extracellular vesicles
ECMO	Extracorporeal membrane oxygenation
ARDS	Acute respiratory distress syndrome
CRISPR	Clustered regularly interspaced short palindromic repeats
IDO	Indoleamine 2,3-dioxygenase

1 INTRODUCTION

Gene and cell therapy in regenerative medicine

Gene and cell therapy are overlapping fields of biomedical research aiming at alleviating or curing disease by means of genetic or cellular transfer. There are also increasing therapeutic strategies combining the two fields in order to improve the therapeutic outcome.

1.1 Gene Therapy

Historically, the development of DNA recombinant technology in the 1970s provided new therapeutic options and laid the foundation for gene therapy. Early in the 70s there were two papers that proposed the theory that disease with "Bad DNA" could be cured with exogenous replacement of "Good DNA" (1, 2). Already in Friedmann´s and Roblin´s 1972 paper (2) fundamental barriers for gene delivery were discussed and outlined as well as discussions on viral and non viral delivery of genetic material. Many of these points are still valid today. This gave rise to a long scientific struggle of vector optimization in order to successfully transfer new therapeutic genes into recipient cells. In 1990 the technology had matured enough to conduct the first clinical experimentation. The affected patients suffered from adenosine deaminase deficiency a congenital disease that gives rise to a severe impairment of immunity and are thus prone to infections. These first clinical trials utilized viruses as transfer vectors due to their superior ability to transfer genetic material into target cells. The results were encouraging but only gave a transient effect on the immune reconstitution (3). Inspired by these findings numerous clinical trials were started but with the death of Jesse Gelsinger, a boy suffering from ornithine transcarbamylase deficiency, and who was enrolled in a gene

therapy clinical trial, many trials were put on hold or stopped. Post mortem autopsy concluded that the cause of death was systemic response against the adenoviral vector administrated in the trial (4). This was followed by an initially promising result of full correction in two patients with X1-SCID, a disease that similarly to ADA gives rise to an immunodeficiency (5). The trial was later put on hold when a number of patients showed tumor development caused by the gene therapy vector (6). Over the years new vectors with a safer profile have been developed and viral-mediated gene therapy has a shown to have very good therapeutic effects in several different condition such as primary immunodeficiencies, hemophilia, cancer and eye disorders (7-11). Interestingly, the development of a leukemia-like condition in patients with X1-SCID has never been observed in patients with ADA-SCID, demonstrating that other parameters than the vector are also important (12).

1.2 Gene therapy principles

Gene therapy can be carried out using three principle strategies namely, gene addition, gene correction and gene knock-down. Gene addition is the most commonly used strategy in gene therapy. In principle, a missing gene due to genetic mutation or overexpression of a transgene is yielding the therapeutic effect. Genetic correction is possibly the most challenging approach from a technical standpoint. It has gained popularity by introduction of zinc-finger nucleases and DNA recombination technologies and has been used to create mutations in CCR5 to prevent HIV infection (13). Especially the recent development of the CRISPR/Cas 9 technology has already had a major impact on the field (14). Gene knockdown is built around the Nobel Prize winning discovery of RNA interference (15). It takes advantage of different RNA species ability to down regulate gene expression and has together with gene correction the greatest potential for optimal clinical results

1.3 Technical barriers to successful gene therapy

Gene therapy is conceptually built on vectors that deliver the genetic material. The various vectors´ history, creation and use are an entire research field of its own. During the last decade improvements in gene transfer into cells or tissue has largely been dependent on advances in vector technologies by refining the vectors systems, enhancing transduction/transfection efficiencies and improving production and safety profiles of the vectors. Although progress has been made there are still hurdles to improve and overcome. The main barriers for delivery of genetic material are described below and in **Figure 1.**

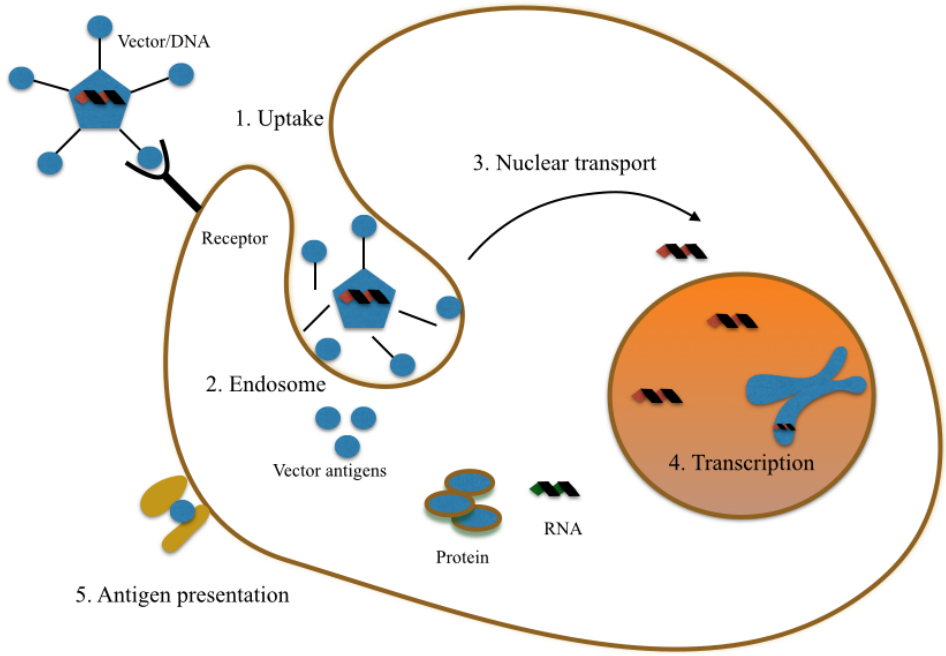

Figure 1. The main barriers for gene therapy

1, Uptake, transport and uncoating of the vector. The vector binds to a cellular membrane and is internalized by different mechanisms. Most uptake steps involve a ligand and receptor interaction. **2**, Most vectors enter the endosome and needs to escape in order to avoid degradation. **3,** Transport to the nucleus is also required for successful therapy and can be achieved during cell division or by active transport. **4,** Vector genome is either integrated or transcribed extra chromosomal. **5,** The immune response against the vector or the transgene product can limit the viability of the transduced cells.

1.3.1 Cellular uptake, transportation and uncoating of vector

In order to mediate the therapeutic effect, a gene therapy vector that is administered by local or systemic delivery needs to be taken up by the target tissue. Vector distribution is influenced by many factors such as vascular supply, endothelial barriers, vector size and ligand receptor interactions. Viruses have by evolution developed efficient mechanisms to enter the cell and the nucleus, but for non-viral vectors the limiting steps are usually endosomal escape and transportation through the nuclear membrane.

1.3.2 Duration of transgene expression.

Vectors can be expressed either as episomes or integrated in the host genome. Episomal vectors will be lost with cell division but can have persistent expression for many years in organs with quiescent tissue such as liver or muscle due to that cell divisions are rare (16, 17). Vectors that integrate into the host genome have sustained expression even in rapidly dividing cells but can potentially activate or disrupt nearby genes by insertional mutagenesis (6). Both episomal and integrated vectors can be silenced through epigenetic modifications of the vector genome. Ideally one should match the duration of the vector expression to the time the vector needs to be expressed to treat a specific disease. Many monogenetic diseases need lifelong treatment that can be achieved either by stable duration of vector expression or repetitive dosing. In other diseases such as cancer a shorter transient expression could be favorable.

1.3.3 The host immune response.

Both the vector and the product of the transgene could potentially activate an immune response. Immune responses can be divided into three types, innate, and adaptive, which can be further subdivided into humoral and cell-mediated immune response. Innate response can cause local or systemic toxicity. Innate response has been a key issue in gene therapy strategies using small RNA molecules (18). Humoral response can be either directed against the vector limiting effectiveness or rule out the possibility of re-administration (19, 20). Humoral response towards the transgene product can also be problematic but avoiding expression of the transgene in antigen presenting cells can lower the risk. Finally, cell mediated immune response can eliminate the transduced cells, the response can be directed against the vector as well as the transgene (21)

1.4 Non viral gene therapy

The early successful protocols in gene therapy utilized viral vectors for transfer of the therapeutic gene. Although the use of viral vectors have advanced the field of gene therapy they have inherent problems in immunogenicity, carcinogenesis, limitation of transgene size, and complex vector production (17, 22-24).. Non viral delivery platform has the potential to address these limitations, especially in respect to safety. The non viral vectors have lower immunogenicity and patients have no pre-excising immunity. Non viral vectors are generally easier to produce and are less restricted regarding the transgene size, comparison of vectors are outlined in **Table 1** (25, 26). As DNA/RNA molecules are too large, hydrophilic and negatively charged to diffuse across cell membranes on their own, delivery vectors or chemical modifications are generally required to bring the therapeutic genetic material to the site of action. This means to the nucleus for plasmids that need to be transcribed. If the genetic drug is a RNA species the site of action is in the cytosol. Before

the target cell can be reached, the systemically administered genetic material needs to overcome a wide range of challenges in the body. The hurdles to overcome are degradation by serum endonucleases, evasion of immune detection, and prevention of nonspecific interactions with plasma proteins or non-target cells, avoidance of renal clearance, ability to extravasate to reach target tissues and promote cell entry. When the target cell is reached the genetic drug needs to escape endosomal degradation and facilitate nuclear entry at cell division or by active transport as described above. Methods developed to overcome barriers for genetic drug delivery include chemical modification, direct injection and the use of nanoparticles as carriers.

Vector	**Cell type**	**Efficacy for in vivo gene delivery**	**Duration of expression**	**DNA carrying capacity**	**Immunogenic response against the vector**	**Production**
Viral	Dividing or non-dividing	High	Stable or transient	<8 Kb	Low to high	Difficult and expensive
Non Viral	Mostly dividing cells	Low	Mostly transient	>10 Kb	Low (mainly) to moderate	Easy and not expensive

Table 1. Gene therapy vector characteristics

1.5 Peptide based gene transfer

Non viral delivery strategies are usually based on complexation of chemical components that form complexes with DNA. One such strategy has been to utilize cell penetrating peptides (CPPs) that form complexes with DNA by noncovalent electrostatic- and hydrophobic interactions. CPPs have been successfully used for carrying different cargoes such as plasmids (pDNAs), oligonucleotides and proteins (27). Although CPPs have been

reported to enter the cell by direct translocation across the cell membrane, most CPPs enter the cell through endocytic pathways. After the compound is internalized into the endosome the complex needs to escape degradation. To overcome this problem many strategies aiming at increasing endosomal escape have been investigated (28, 29). One possibility to increase the activity of CPPs is to introduce a stearyl moiety. This approach has previously been reported to improve polyarginine-mediated delivery of small interfering RNAs and plasmids (30-33). However, the stearylation strategy does not seem to provide the same effects for all peptides.

1.6 Cell therapy

Allotransplantation of whole organs has been the classic way to restore damaged organ function. The first transplantation was done by Theodor Kocher that performed a thyroid transplantation in 1883 and he was later awarded the Nobel prize in medicine for his groundbreaking studies. Today organ transplantation has matured and transplantation of organs such as heart, kidney, liver, lungs and intestines are carried out on a routine basis. Although effective, the method is limited due to shortage of organs available for transplantation and possible immune rejection of allotransplants. The adaptive and innate immune systems, mentioned earlier, are the human organism's defense systems against pathogens resulting in that essentially all transplantations from non-identical individuals end in rejection of the grafted cells or organs if not prevented by immunosuppression. Immunological rejection can be divided into three different categories that are ABO blood group antigen, major histocompatibility complex, and minor histocompatibility complex rejections (34)

Cellular therapy comprises of a broad spectrum of strategies in which cells are used in order to cure or alleviate disease. Apart from blood transfusions and skin transplantation the first

successful use of cells as treatment was done in 1953 by Mathe et al where they demonstrated grafting of chimeric bone marrow in a patient accidently exposed to whole-body irradiation. They subsequently confirmed the usefulness of this strategy in patients with leukemia that had undergone irradiation therapy and could in certain individuals reconstitute hematopoietic function by allogenic bone marrow engraftment. (35, 36) These early experiments have developed into accepted clinical practice of a number of hematological disease including malignancies, immune deficiencies and inborn errors of metabolisms to name but a few. In later years cellular based therapies has focused on the use of stem or progenitor cells to amend disease and recover organ function. There are two principal therapeutic mechanisms. The first comprises of progenitor or multipotent stem cells for the long-term regeneration of damage tissue or function. The second category is that the transplanted cells mediate the therapeutic effects by the release of soluble factors such as cytokines, growth factors and exosomes. Messenchymal Stromal Cells (MSC) used in treatment of Graft vs Host disease folds into this category (37, 38). In later years much research has been done on embryonic stem cells and induced pluripotent stem cells that falls into category number one. Schwartz et al. reported data on two prospective phase 1/2 studies on subretinal transplantation of hESC-derived retinal pigment epithelium into 18 patients with macular dystrophy. The underlying pathology is caused by degradation of macular cells due to genetic mutations in the retinal cells leading to blindness. The initial results are promising showing, no evidence of adverse proliferation; transplant rejection or any other serious safety issues regarding the transplanted cells. In 10 out of 18 patients there is a significant improvement in eye function, seven patients have experienced a modest improvement and only one patient has suffered a decline in the eye function (39). Similarly a Japanese woman in her 70s with wet type age-related macula degeneration became the first patient to receive tissues derived from human iPSCs in September 2014. The underlying pathology is caused by damage of the photoreceptors in the eye leading to blindness. The retinal pigment epithelium cells given as an injection into the

eye had been derived from autologous human iPSC line generated from the patient's own skin cells. No results from the trial have been reported to date.

Regardless the cell type or disease several hurdles need to be over come in order to develop cell therapy to mainstream clinical treatments. These hurdles include generating sufficient number of cells, development of xeno-free culturing methods, reducing tumorgenicity and safety issues as well as overcoming immunological barriers to name a few (40).

1.7 *In vitro* culturing of human cells

Degenerative diseases, such as Parkinson's disease, diabetes, congenital eye disease and others, as well as severe injuries either completely eliminate entire cellular populations or significant fraction of them thus leaving no cellular target for standard medical treatments. Therefore, cellular transplantations are warranted to restore function and structure of the affected organs

As a part of a multicellular organism, almost any mammalian cell is intricately controlled by molecular cues of the extracellular milieu that include interactions with other cells, extracellular matrix molecules, and soluble factors from the bodily fluids. Therefore, *in vitro* culturing environment must provide mammalian cells with essential signals to stay alive, proliferate, and preserve (or change if it is needed) cellular identity. Mimicking the natural cell environment inside the body is in theory a straightforward way to achieve this goal. For instance, extracellular matrix (ECM) proteins that are in contact with certain cells may be studied using immunofluorescence methods and appropriate ECM molecules may be later used as cell substrata to culture those cells *in vitro*.

1.8 Laminins

One important class of ECM proteins is laminins (41). They are large heterotrimeric glycoproteins containing one α, one β, and one γ chains. Laminins are named after their chain composition, for instance laminin-221 (LN-221) consists α2, β2, and γ1 chains (42). Five α chains, four β chains, three γ chains, and 16 combinations of them have been discovered in humans to date (41). Mutations in laminin chains often lead to embryonic lethality or severe diseases (41). Laminins (LNs) are the major part of basement membranes (43). The ECM glycoproteins are in direct contact with the majority of cell types *in vivo* often inducing signalling inside cells via interaction with cell membrane receptors. Integrins (44-46), dystroglycan (45, 47), Lutheran receptor (48, 49) and sulfated glicolipids (50) are cell membrane receptors that bind various laminin isoforms. Apart form direct signalling, laminins are capable of co-signalling with other ligands of various kind and polarization of cells thus significantly affecting cellular behavior (41). Laminins affect migration, proliferation, differentiation, and survival of various cells in the human body. Expression of laminins display various spatio-temporal patterns and certain degree of tissue specificity (41), for instance LN-211 is specific for muscle and neural tissues, LN-332 is specific to epithelia.

1.9 Sources of cells for regenerative medicine

Somatic cells isolated from humans undergo senescence with time in culture that limits their proliferation potential. Majority of somatic cells expresses high levels of human leukocyte antigens (HLAs). Expression of HLAs confers immune rejection of cells after transplantation into genetically non-identical individuals (allotransplantations). Therefore, somatic cells are not a preferred source of cells for regenerative medicine, especially if the aim is longterm engraftment. Various human pluripotent stem cells (hPSCs) exhibit essentially unlimited proliferation potential and can provide an almost unlimited number of cells for regenerative

medicine (40). Multipotent mesenchymal stromal cells do not induce alloreactive response, at least not to a substanial degree. Therefore, these two types of cells are promising sources of cells for regenerative medicine.

1.10 Human pluripotent stem cells

All hPSCs share abilities to proliferate indefinitely and to give rise to all cellular lineages in the body. Several types of hPSCs have been described to date (51-54). Human embryonic stem cells (hESCs) were first derived in 1998 (51). Human induced pluripotent stem cells (hiPSCs) were developed later (52, 55), but they are the most commonly used in the research laboratories type of hPSCs at the moment. Development of hiPSC-based clinical treatments have been hampered by epigenetic abnormalities as well as other factors difficulties (40).

The ability to proliferate indefinitely, differentiate into all somatic cellular lineages, and low rate of epigenetic abnormalities are the main advantages of hESCs that are still considered as 'gold standard' hPSCs and are already used in several clinical trials (39, 40). Since hESCs are usually derived from the inner cell masses of human blastocysts, they are ethically controversial and allogeneic to patients. Only supernumerary *in vitro* fertilized (IVF) blastocysts that cannot be used in infertility treatments with informed consent of the both donors are used to derive new hESC lines (56). Nevertheless, the procedure demands destruction of human embryos, which is an important ethical concern. Derivation of hESC lines from a single cell (blastomere) biopsy of a human 8-cell embryo can address the ethical concern of many (57). Acquisition of a blastomere does not interfere with developmental potential of the embryo and is a standard procedure that is used in *in vitro* fertilization clinics during treatment of pairs with certain genetic backgrounds.

Although different in some important details, hPSCs exhibit many common features and are cultured using same methods. In the past, hPSCs were cultured on a layer of feeder cells (51) (usually murine or human fibroblasts) or on Matrigel (58). The latter is a protein preparation from Engelbreth–Holm–Swarm mouse sarcoma. Matrigel is a complex mixture of ECM proteins and growth factors that are bound to them. Both feeders and Matrigel are batch-to-batch variable, chemically undefined substrata that pose a risk of contamination with infectious adventitious agents or induction of undesired immunogenicity in hPSCs cultured on them. Therefore, culturing of hPSCs involving feeder cells or Matrigel is unreliable and may be unsafe. Preferably, development of cells aimed at therapeutic applications should be done under chemically defined and animal product-free (xeno-free) conditions. Several such methods, which may significantly facilitate development of clinical treatments involving hPSCs, have been reported to date (59-62).

1.11 Multipotent mesenchymal stromal cells

Multipotent MSCs are isolated from bone marrow, fat and other tissues (63). At the moment they are defined by the ability to adhere to cell-culture treated plastic, expression, or lack of expression of certain markers (CD73+, CD90+, CD105+, CD11b-, CD14-, CD34-, CD45-, CD19-, CD79-a, and HLA-DR-) and the ability to differentiate into adipocytes, osteoblasts, and chondrocytes *in vitro* showing multipotency (64). Importantly, individual mesenchymal stromal cells exhibit different levels of multipotency suggesting heterogeneity of MSC populations (65). Apart from multipotency, MSCs exhibit strong immunomodulatory properties (63). This feature has given rise to many preclinical and clinical studies on treatment of various immune and inflammatory diseases with MSCs (38, 66-68). In many cases, the therapy has shown significant efficacy but the exact molecular mechanism of the action is not entirely clear. Although MSCs are capable of homing to sites of injury, there is

little proof for long-term incorporation into the recipient's tissues suggesting that paracrine mechanisms are the main mode of action. Antidonor immune responses in allo-MSC transplantations have been reported in preclinical models (69) and can potentially explain the fast clearance and low engraftment (69). However no adverse events has been reported related to an antidonor immune response in clinical trials (70).

Interestingly, MSC may exhibit both immunosuppressive and immunoactivation properties depending on signals from the extracellular milieu (71). It has been surmised that circulating factors in the particular patient at the time of infusion define pro- or anti-inflammatory properties of the MSCs (72) . Toll-like receptors on the MSC surface can bind bacterial products such as lipopolysaccharide (LPS) from the local milieu and induce expression of pro-inflammatory factors TNFα, IL-6, IL-8, and others (73) by the MSCs. On the other hand, the immunosuppressive phenotype of MSC is induced by contact with pro-inflammatory cytokines from the environment (74). In human MSCs, INFγ–induced expression of indoleamine 2,3-dioxygenase (IDO) enzyme has been shown to mediate inhibition of T-cells by inducing apoptosis in them (75, 76). Other mechanisms that do not rely of IDO expression have been also described (77). Expression of prostaglandin E_2 (PGE_2) by MSCs in response to inflammatory cytokines IFNγ and TNFα has been shown to promote both pro- and anti-inflammatory effects depending on the PGE_2 concentration and other factors from the local environment (78, 79). An important example is the induction of T regulatory cells, which are pivotal for suppression of immune reactions, by PGE_2 expressed by MSCs (80). Another interesting mediator is Human leukocyte antigen-G (HLA-G), known for its tolerogenic and potent immune inhibitory function, exists in seven different isoforms, of which the full-length transmembrane HLA-G1, and its soluble counterpart HLA-G5, are the most extensively studied (81, 82). Both HLA-G1 and HLA-G5 are potent suppressors of allogeneic T-cell response through induction of CD8+ T-cell apoptosis and arrest of T- and B-cell proliferation, inhibitors of natural killer cell cytotoxicity, they can also induce T

regulatory cells (83) (84). MSC have been shown to constitutively express HLA-G at low levels (83). Recently, microRNAs (miRNAs) have been identified as an additional factor of the milieu that may affect expression of various proteins in MSCs or may be released by MSCs (85).

Paracrine factors that are released by MSCs may affect both nearby cells and cells situated at large distances. Extracellular vesicles (EV) including exosomes that are small structures encapsulated into lipid bi-layer and released by cells mediate the delivery of the paracrine factors at large distances (86). EVs have been shown to interact with cell receptors via molecules on their surfaces and to merge their content with the recipient cells thus delivering the paracrine factors. Interestingly, MSC-EVs themselves, without MSCs of origin, have exhibited the therapeutic potential in various preclinical studies (87). **Figure 2** outlines some of the factors and cells MSC are believed to affect to yield a therapeutic response.

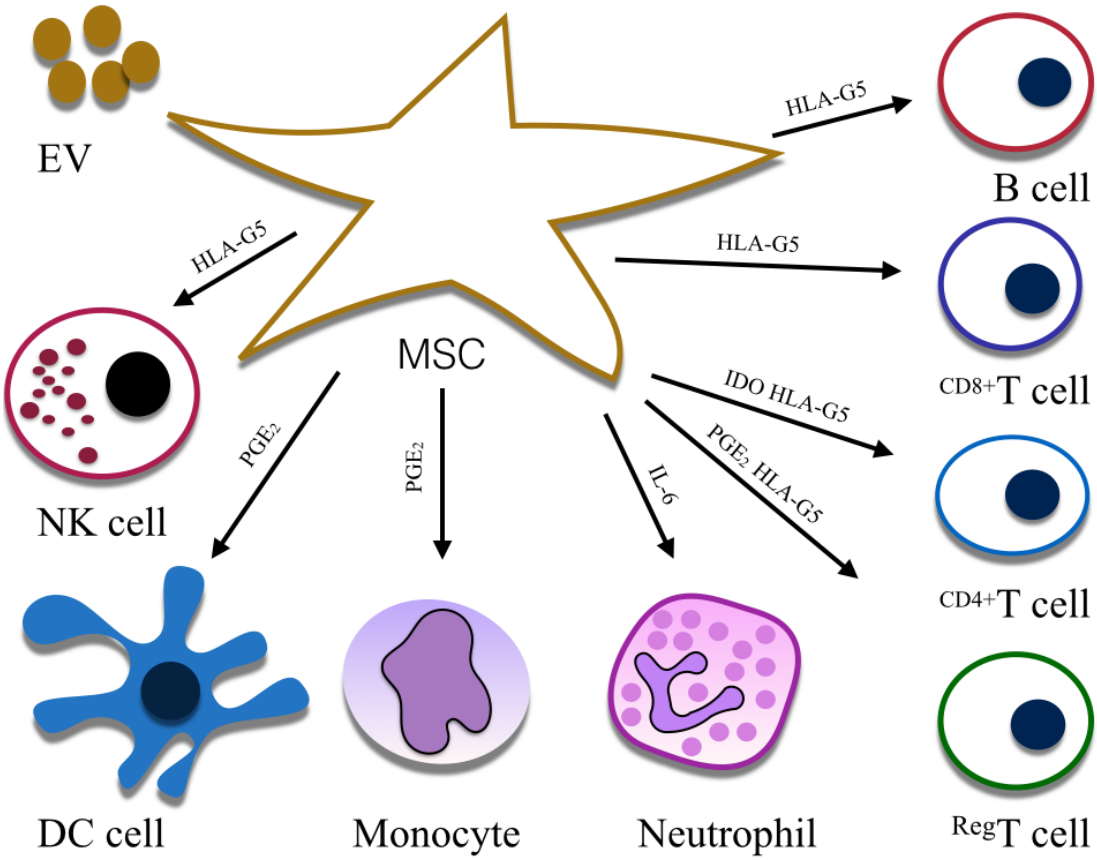

Figure 2. **MSC mechanistic environment**

It is a complex mechanistic environment that is involved in MSCs' therapeutic response. This involves soluble factors as IDO, PGE, HLA-G5, IL-6 and extracellular vesicles that affect immune cells.

1.12 Extracorporeal membrane oxygenation

Extracorporeal membrane oxygenation (ECMO) is a technique to provide advanced support to patients with heart and lung failure. The ECMO circuit is removing blood from the patient's body and removes carbone dioxide and oxygenates the red blood cells before pumping the blood back to the patient. The technique was develop from Gibbons heart and lung machine and was first demonstrated in infants undergoing heart surgery for congenital heart defects (88, 89). In 1976 the first report on successful use of ECMO in respiratory failure was demonstrated in neonates with severe respiratory distress (90). Although

primarily developed for neonatal support ECMO has become more popular as support therapy in the adult population. The two most common types of ECMO are veno-venous (VV) and veno-arterial (VA) ECMO. In both types the blood is drained from the venous site and in VV ECMO the blood is returned on the venous site whereas in VA ECMO the blood is returned to the arterial site. The use of VV ECMO is more popular in pulmonary failure and VA ECMO more in heart failure.

1.13 Acute respiratory distress syndrome

Acute respiratory distress syndrome is caused by direct or indirect damage to the lungs. Direct causes are viral or bacterial pneumonia, aspiration pneumonia, chest trauma or inhalation of toxic fumes. Indirect causes comprise of systemic processes such as sepsis, pancreatitis, serial transfusions or multiple trauma. Diagnostic criteria using the Berlin definition is presented in **Table 2**. Although debuting with respiratory failure it can develop into a multiorgan failure affecting other organs such as the liver and kidney. A number of different pharmacologic therapies have failed to demonstrate benefit and treatment is currently limited to supportive care using low tidal volume mechanical ventilation and fluid management (91, 92). The addition of extracorporeal membrane oxygenation (ECMO) has been used in patients with severe ARDS, however, in a recent systematic review and meta-analysis of current evidence, no association with improved outcomes could be demonstrated in adult patients (93).

ARDS is characterized by severe acute inflammatory response in the lung. This gives rise to damage of the alveolar-capillary barrier and the resulting oedema hampers the gas-exchange. Early mortality in ARDS is frequently caused by hypoxia/anoxia and after two weeks the mortalities are more attributed to pulmonary fibrosis, nosocomial infections and multiorgan failures (94). The pathology is not fully understood, but what is known is that early on there

is a loss in capillary endothelial cells as well as type 1 alveolar cells and protein rich oedema is then flooding the alveolar and interstitial space .The epithelium is then replaced by hyaline membranes. Neutrophils invade the damaged tissue and adhere to the activated endothelium and polymorphonuclear leukocytes/neutrophils further damage the lung with the release of proteolytic enzymes, oxidants and reactive nitrogen species. The release of chemokines also induces the migration of macrophages that release cytokines and apoptosis inducing ligands. Within 3 days the secondary fibroproliferative phase starts with infiltration of fibroblast as well as of mesenchymal progenitor cells. Loss of type 1 pneumocytes occurs and this cell type is replaced by type 2 pneumocytes and fibrin is accumulated due to reduced fibronolytic activity. The matrix composition is also changed with replacement of type III collagen with type I collagen. This leads to a fibrotic and stiff lung (95). There is currently no animal model for ARDS but there are several models of acute lung injury, the most common ones being based on lipopolysaccharide or Bleomycin..

Non-HLA matched, mesenchymal stromal cells (MSCs) have been demonstrated to be safe and potentially effective in different clinical applications (96-99). In several preclinical models of acute lung injury MSCs have demonstrated therapeutic potential (96, 100-106). While not fully understood, the mechanisms of MSC actions in acute models of ARDS include the release of paracrine anti-inflammatory and anti-bacterial peptides and mitochondrial transfer through cell-cell contact with damaged alveolar epithelial cells (102, 107-109). Additionally, MSCs release extracellular vesicles that can reduce inflammation and promote tissue regeneration in different pre-clinical models (110-113). Due to the beneficial safety profile of non-HLA matched allogeneic MSCs in combination with scientific publications demonstrating promising results in a widening range of clinical applications in both lung diseases as well as other diseases (96-99), there is a growing interest in MSC treatment for ARDS. Recently a phase 1 dose-escalation study demonstrated safety of a

single intravenous administration of 1 to 10 million cells/kg of MSCs in 9 patients with moderate to severe ARDS and a phase 2 trial is currently underway (114).

ARDS criteria according to the Berlin definition
Lung injury of acute onset, within 1 week of an apparent clinical insult and with progression of respiratory symptoms
Bilateral opacities on chest imaging not explained by other pulmonary pathology
Respiratory failure not explained by heart failure or volume overload
PaO_2/FiO_2 ratio: mild ARDS: ratio 201 – 300 mmHg moderate ARDS: ratio 101 – 200 mmHg severe ARDS:ratio $\leq$ 100 mmHg With a positive end-expiratory pressure (PEEP) $\geq$5 cm H_2O

Table 2. ARDS diagnostics according to the Berlin criteria

2 MATERIALS AND METHODS

Ethical considerations.

Project 1 was done under the ethical approval N310/08 and N309/08 for the use of labaratory animals.

Project 2 was done under the etical approval S198-11 for the use of labaratory animals and propageting hESCs Dnr 454/02.

Project 3 The production of clinical-grade MSC was accredited by the Swedish Board of Health and Welfare (Approval # 9.1-57237/2012) and the Medical Products Agency (Approval number: 5.9.2-2013-047346). ECMO is a last resort supportive therapy for ARDS. The patients in this report deteriorated on ECMO support and since the patients were sedated prior to MSC administration, informed consent could not be obtained. At a multidisciplinary conference the decision was made to use MSCs on a compassionate use basis under the approval of the chief medical officer of the hospital, the hospital ethics committee, and the relatives of the patients. A phase 1 trial is on the way with the ethical approval number DNR 2013/1908-31/2 and Clinicaltrial.gov identifier NCT02215811,

Paper I

Synthesis of peptides.

All peptides were synthesized using an automated peptide synthesizer (ABI433A; Applied Biosystems). N-terminally stearylated peptides were prepared by treatment of peptidyl-resins with 4 eq. stearic acid (Sigma) and 4 eq. HOBt/HBTU (MultiSynTech) and 8 eq. DIEA (Fluka). Peptides were purified by reversed-phase high-performance liquid chromatography. The molecular weight of the peptides was analyzed by matrix- assisted laser desorption/ionization-time of flight mass-spectroscopy and purity was >90% as determined by analytical high-performance liquid chromatography.

Cell culture. CHO cells were grown at 37 °C, 5% CO_2 in Dulbecco's modified Eagle's medium F12 with glutamax supplement with nonessential amino acids, sodium pyruvate, 10% fetal bovine serum, penicillin, and streptomycin (PAA Laboratories GmbH).

HEK293, U87, U2OS and mouse embryonal fibroblast cells were grown at 37 °C, 5% CO_2 in Dulbecco's modified Eagle's medium with glutamax supplemented with nonessential amino acids, sodium pyruvate, 10% fetal bovine serum, penicillin, and streptomycin (PAA Laboratories GmbH).

Complex formation. 0.5 μg of pGL3 or pEGFP-C1 plasmid (4.7 kb), expressing luciferase or EGFP respectively, was mixed with CPPs at different peptide:plasmid CRs of 0.5:1–4:1 (CR0.5–CR4) in milli-Q water in 50 μl (1/10th of the final treatment volume). CRs were calculated theoretically, taking into account the positive charges of the peptide and negative charges of the plasmid. Complexes were formed for 1 hour at room temperature. Meanwhile, cell medium was replaced in 24-well tissue culture plates for fresh media (450 μl). In case of LF2000 (Invitrogen), the complexes were formed according to the manufacturer's protocol, using the recommended amounts for each cell line. Additional luciferase- expressing plasmids were used, namely pcDNA4/TO-Ubi-FFLuc (7kb) and pEGFPLuc (6.4 kb), both from Clontech.

DNA condensation was analyzed using an EtBr (Sigma, Taufkirchen, Germany) exclusion assay. Briefly, complexes were formed as described above. After 1 hour incubation, 135 μl milli-Q water was added to each sample and transferred into a black 96-well plate (NUNC). Thereafter, 15 μl of EtBr solution was added to give a final EtBr concentration of 400nmol/l. After 10 minutes, fluorescence was measured on a Spectra Max Gemini XS fluorometer (Molecular Devices, Palo Alto, CA) at λ_{ex} = 518 nm and λ_{em} = 605 nm. Results are given as

relative fluorescence and a value of 100% is attributed to the fluorescence of naked DNA with EtBr.

Stability of stearyl-TP10/plasmid nanoparticles was evaluated in the presence of serum. Briefly, complexes were formed as described above. Thereafter, serum was added to the complexes at standard concentration (10%) and incubated over different periods of time. At 0, 1, 2, 4, and 24 hours samples were loaded on an agarose gel (2%) and imaged by staining the gel with EtBr (0.5 μg/ml).

Dynamic light scattering and zeta potential measurements.

Hydrodynamic mean diameter of the DNA nanoparticles was determined by dynamic light scattering studies using a Zetasizer Nano ZS apparatus (Malvern Instruments). pDNA complexes resulting from the addition of stearyl-TP10 were formulated according to the protocol for *in vitro* transfection, as described above, and assessed in disposable low volume cuvettes. Briefly, pDNA complexes were formulated in deionized water, in 20 μl volume, at a final concentration of 0.01 μg/μl of pDNA. After 30 minutes incubation at room temperature, the DNA complexes were diluted in Opti-MEM into a final volume of 200 μl. All data was converted to "relative intensity" plots from where the mean hydrodynamic diameter was derived. æ-Potential was measured in Opti-MEM supplemented with 10% fetal calf serum. Measurements were performed in ZS Malvern instrument.

Heparin displacement assay.

For the analysis of their resistance to heparin, peptide formulations containing 100 ng of pDNA were incubated for 30 minutes at 37 °C in the presence of heparin sodium (Sigma-Aldrich) over a range of concentrations. After the incubation period, loading buffer was added and the reactions were analyzed on 0.8% agarose gels in 1× TAE buffer and visualized by staining with SYBR Gold (Invitrogen). Gels were documented using the Fluor-S system.

Plasmid delivery assay.

CHO, HEK293, U87, U2OS, and mouse embryonal fibroblast cells were seeded 24 hours before experiment into 24-well plates. Cells were treated with CPP/plasmid complexes at different CRs for 4 hours in serum-free or serum containing media followed by addition of 1 ml 10% serum containing medium and incubated for another 20 hours. Thereafter, cells were washed and lysed using 100 µl 0.1% Triton X-100 in HEPES-buffered Krebs Ringer buffer at room temperature. Luciferase activity was measured using Promega's luciferase assay system on GLOMAX 96 microplate luminometer (Promega) and normalized to protein content (Lowry; Bio-Rad). LF2000 (Invitrogen) was used according to the manufacturer's protocol and results were taken as a positive control for measuring transfection efficiency.

In experiments with chloroquine, after complex formation and before treatment of cells, chloroquine was added to the complex solution. Four hours after addition of the complexes and chloroquine to cells, cell medium was replaced with fresh medium in order to avoid toxicity effects.

Spectrofluorometry analysis.

U2OS cells were seeded in 24-well plates 24 hours before cellular treatments with fluorescein-plasmid (Mirus) complexed with TP10 or stearyl-TP10 as described in the complex formation section. Cells were treated for 24 hours either in Opti-MEM or in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were washed twice with phosphate-buffered saline and once, briefly, with trypsin to remove membrane-bound complexes. Cells were thereafter lysed using 0.2% Triton in phosphate-buffered saline for 1 hour and lysates were transferred to a black 96-well plate. Fluorescence was measured on at 490/518 nm on a Spectra Max Gemini (Molecular Devices) fluorometer. Fluorescence signal (RFU) from untreated cells was subtracted from the signals of treated

cells.

Confocal microscopy.

CHO cells were seeded 24 hours before experiment onto 13 mm tissue culture coverslips that were placed into a 24-well plate. Cells were treated with pEGFP-C1 plasmid and CPP complexes at different CRs (1–3) for 4 hours in serum-free media followed by addition of 1 ml of full growth media and incubated for another 20 hours at 37 °C. LF2000 (Invitrogen) was used according to the manufacturer's protocol. Thereafter, cells were washed with phosphate-buffered saline and fixed by using 4% formaldehyde solution. Images were captured using 60-fold objective on Nikon Eclipse TE2000-U inverted microscope and a digital camera DXM1200C, and processed with EZ-C1 software V.2.30 (Nikon).

WST-1 proliferation assay.

Cell proliferation was studied with the Roche WST-1 proliferation assay according to the manufacturer's instructions. Briefly, cells were seeded one day before the experiment in a 96-well plate. Cells were treated with stearyl-TP10/plasmid nanoparticles at different CRs for 4 hours in serum-free medium followed by addition of 10% serum containing medium and incubated for another 20 hours. Transfection with LF2000 was carried out according to the manufacturer's protocol. WST-1 was added according to manufacturer's protocol (Roche Diagnostics Scandinavia AB). WST-1 measures the activity of mitochondrial dehydrogenases to convert tetrazolium salts to formazan, which absorbs light at 450nm. Absorbance was measured on Digiscan absorbance reader (Labvision *via* AH Diagnostics AB). Untreated cells were defined as 100% viable.

IL-1β, tumor necrosis factor-α, and IL-6 analysis.

THP1 cells were differentiated using phorbol myristate acetate for 48 hours and subsequently seeded into 24-well plates. Cells were treated as previously. Lipopolysaccharide was used as

positive control. Culture supernatants were collected at 4 hours and 24 hours after treatment, and assayed for IL-1β and tumor necrosis factor-α by enzyme- linked immunosorbent assay according to manufacturer's protocol (R&D systems). IL-6 and tumor necrosis factor-α levels in blood were analyzed at 24 hours post i.d. or i.m. treatments of NMRI female mice. Blood was collected retro-orbitally and serum was purified using serum separation tubes (BD Bioscience). An amount of 100 μl serum was assayed using enzyme-linked immunosorbent assay Max Deluxe Set (BioLegend) and absorbance measured on Spectra Max Gemini (Molecular Devices).

In vivo experiments.

Female Balb/c mice were first anaesthetized with isoflurane gas and kept under anesthesia during the administration procedure. For the complex formation, 1, 5, or 10 μg of pGL3 (4.7 kb) or pEGFPLuc (6.4 kb) plasmid was mixed with stearyl-TP10 at CRs of 0, 0.5, 1, and 2 in 5% glucose using a total volume of 50 μl. In decay kinetics measurements 5μg of pGL3 plasmid was used, while in dose-dependency experiments, the abovementioned doses of pEGF- PLuc plasmid was used. Thereafter, stearyl-TP10/plasmid nanoparticles were injected i.d. or i.m. into *M. tibialis anterior*. Gene expression was assessed by imaging of the reporter gene (firefly luciferase) expression. Anesthetized mice were injected intraperitoneally with 150 mg/kg of D-Luciferin (Xenogen). Light signals (CCD) images were obtained using an IVIS 100 system (Xenogen). Luciferase expression was quantified by total flux using Living Image Software (Xenogen). The maximum photon/second of acquisition/cm^2 pixel/steridian was deter- mined within a region of interest to be the most consistent measure for comparative analysis. In general, acquisition times ranged from 10 seconds to 1 minute.

Clinical chemistry and histopathology.

Clinical chemistry parameters (alanine transaminase/aspartate transaminase, C-reactive protein, and creatinine levels) in serum from Balb/c mice were analyzed after 24 hours post-treatments by the Clinical chemistry laboratory at Karolinska University Hospital using International Federation of Clinical Chemistry and Laboratory Medicine standardized techniques. Blood was collected retro-orbitally and serum was purified using serum separation tubes (BD Bioscience). Organs were dissected after 24 hours and fixed in formalin, embedded in paraffin, and stained with eosin and hematoxylin. Images were taken on the Olympus BX45 microscope with a Sony DXC-S500 digital camera. Histology sections were analyzed by the Department of Pathology at Karolinska University Hospital.

Ethical permission.

The animal experiments were approved by The Swedish Local Board for Laboratory Animals. The experiments were performed in accordance with the ethical permission and were carried out in accordance to European Community directive (86/609/EEC). All animal experiments were designed to minimize the suffering and pain of the animals.

Statistics.

Values in all experiments are represented as mean ± SEM of at least three independent experiments done in duplicate. Increase in delivery efficiency was considered significant at $***P < 0.001$ using analysis of variance Dunnett's multiple comparison test or analysis of variance Bonferroni's multiple comparison test. In toxicity measurements, decrease in viability was considered significant at $***P < 0.001$ using analysis of variance Dunnett's multiple comparison test.

Paper II

Human ES and iPS cell cultures

All the earlier established hES cell lines and the human iPS ChiPSW cell line were provided by Prof. Hovatta. CVTB1.2 cell line was provided by Dr. Hansson. hES cells and iPS cells were maintained in mTeSR1 (prepared in our laboratory or purchased from STEMCELL Technologies), TeSR2 (STEMCELL Technologies), and NutriStem hESC XF (Biological Industries, Israel) media as described in Supplemental Experimental Procedures. Matrigel (STEMCELL Technologies) and laminin-521 (LN-521) were used as cell attachment substrata.

Cell culture dish coating

Laminins and Matrigel.

Tissue cell culture plates from Sarstedt were coated overnight at 4 °C with sterile solutions of laminins, such as human recombinant LN-521, all at a concentration of 30 μg/ml (5 $\mu g/cm^2$). Laminins were obtained from BioLamina AB and were produced as described in Supplemental Experimental Procedures. Control plates were coated with Matrigel according to the STEMCELL Technologies' instructions.

LN-521/E-cadherin coating.

LN-521 and E-cadherin (R&DSystems) were taken at 9 to 1 w/w ratio with DPBS containg Ca^{2+} and Mg^{2+} used to dilute the proteins. Tissue culture plates were coated at +37 ^{o}C for 2 hours at a concentration of 13.5 μg/ml (2.25 $\mu g/cm^2$) LN-521 and 1.5 μg/ml (0.25 $\mu g/cm^2$) E-cadherin. After that the plates were washed twice with DPBS containg Ca^{2+} and Mg^{2+} and, then, prewarmed stem cell medium of choice was added.

Reagents and antibodies

Function blocking antibodies to various integrin subunits, mouse isotype antibodies, and α-

dystroglycan were purchased from Millipore. Antibodies to Lutheran receptor and α-fetoprotein, as well as rat isotype control antibodies were obtained from R&D Systems. Antibodies to Oct4, Nanog, SSEA-4, smooth muscle actin, and MAP-2 were purchased from Millipore.

Immunofluorescence

For immunofluorescence studies, ES cells were cultured and fixed by 4 % paraformaldehyde, permeabilized, and blocked by bovine fetal serum (GIBCO) for one hour. The samples were incubated with primary antibody washed 3-5 times, incubated with secondary antibody and 4,6-diamidino-2-phenylindole (DAPI, Molecular Probes), and washed 5 times. Specimens were preserved in a fluorescence mounting medium (Dako, Glostrup, Denmark), and observed under a fluorescence microscope (Leica).

Real-time PCR quantification of different mRNAs

Total RNA was isolated using Absolutely RNA Microprep Kit (Stratagene) according to the manufacturer's instructions. cDNA was synthesized using total RNA in 20 μl reaction mixture, containing oligo(dT) primers and Superscript II reverse transcriptase (GIBCO Invitrogen). Real-time quantitative RT-PCR Taqman assays were performed using the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems). All reactions were done in quadruplicates with the use of pre-developed gene expression assay mixes (Applied Biosystems). Additional reactions for each experiment included pre-developed gene expression assay mix for GAPDH for normalizing the RNA input.

FACS analysis

Cells were removed from the culture dish with Trypsin/EDTA, dissociated into single cell suspension and resuspended in ice-cold FACS buffer. Incubation with primary antibodies against SSEA-4 (from Millipore) was performed for one hour on ice. Then, the cells were washed three times with ice-cold FACS buffer. Subsequently, the cells were probed in FACS buffer with 1:400 dilution of Alexa Fluor anti-mouse secondary antibodies (GIBCO)

for 30 minutes in the dark, and washed four times. Control cells were incubated with mouse immunoglobulins and, subsequently, with the secondary antibody as described above. Cells were analyzed on FACSCalibur Flow Cytometer.

Karyotyping

Karyotyping of the cell lines was carried out using standard Q-banding techniques. Samples of cells were treated with colcemid KaryoMAX (0.1 mg/ml; Gibco) for up to 5 h prior the procedure. A minimum of 10 metaphase spreads were analyzed and additional 20 were counted.

In vivo *imaging and migration assay*

The ES cells were plated onto extracellular matrix-coated plates and left to adhere for half an hour. After that, the plates were transferred into a high throughput imaging system (Operetta, PerkinElmer) equipped with an environmental control unit (37 °C, 5% CO_2). The brightfield images were taken once in 30 minutes during several days after plating using Harmony software (PerkinElmer), exported, and analyzed using ImageJ software (NIH, the US).

Teratoma formation

Teratoma formation experiments were done by implantation of approximately 10^6 cells beneath the testicular capsule of a young (7-week-old) severe combined immunodeficiency (SCID) mouse or by injecting the cells subcutaneously in female SCID mice. The mice were sacrificed eight weeks after the implantation. The teratomas were fixed, and sections were stained with hematoxylin and eosin (HE) or with hematoxylin, eosin and PAS (HE-PAS). All animal experiments were performed in accordance with the ethical committee's approval.

Embryoid body formation

ES cells were scraped from cell culture dishes in large cellular clumps and cultured in suspension in low adhesion plates in Knockout DMEM (GIBCO) supplemented with 20%

fetal calf serum (GIBCO). After 1-2 weeks in suspension, the embryoid bodies were transferred onto gelatin coated plates, cultured for 1-2 weeks, then fixed, stained with antibodies against markers of all three embryonic germ line layers (smooth muscle actin, MAP-2 and α -fetoprotein), and analyzed as described above for immunofluorescence.

Cell adhesion assay

Multi-well plates were coated by extracellular matrix proteins as described above. The ES cells were plated onto extracellular matrix-coated plates and left to adhere for 1 hour. Subsequently, the plates were washed 3 times with the medium to remove the non-adherent cells, and then the adherent cells were fixed by 5% glutaraldehyde, stained by 0.1% Crystal Violet (Kebo Lab, Spanga, Sweden). After one hour and 3 washes with water, Crystal Violet was extracted with 10% acetic acid and quantified by measuring optical density at 570 nm. All the experiments were performed in quadruplicates.

Cell survival assays

The survival assay was performed as described for the cell adhesion assay above, except that the cells were left in the cell incubator for 24 hours. For inhibition of the survival assay, the cells were kept in a medium with function blocking antibodies at the concentrations recommended by the manufacturer or pathway inhibitors at concentrations indicated in the text for 30 minutes, and then plated on the coated dishes. All the experiments were performed in quadruplicates.

Western blotting and ELISA

After two washings in ice-cold PBS, plates with cells were snap frozen in liquid nitrogen and stored at -80 °C. To prepare samples for western blots and ELISA, the plates were slowly thawed and kept on ice with 100-150 μl of lysis buffer and Phospho-Stop™ (Roche)) on top. Then, the cells were scraped, pipetted, sheared through a 27G ¾" needle, and clarified by centrifugation. For western blots, 4-12 % gradient gels were used for SDS electrophoresis

and the proteins were transferred to PVDF membranes. The membrane was hybridized with the antibody of interest according to the manufacturer's instructions. Chemoluminescent HRP-substrate was used for visualization. For the densitometry analysis the films were scanned at 2,400 dpi and analyzed by the ChemiImager5500 program. For ELISA the samples were applied to the wells according to the manufacturer's instructions.

Derivation of hES cell lines from whole ICMs of blastocysts

The embryos for derivation of new ES cell lines were obtained as donations from the Fertility Unit of Karolinska University Hospital, Huddinge. Only embryos that could not be used for the couples' infertility treatment were used after informed consent given by both partners of the couples. The work was done in accordance with ethics approval for derivation of new human ES cell lines issued to us by the Regional Ethics Board in Stockholm.

Day 5-7 blastocysts donated after informed consent of both donors, were scored and transferred to TESR2 (Stem Cell Technologies) or NutriStem hESC XF medium. The inner cell masses (ICMs) were isolated mechanically using two sharp metal needles (ICM-tools were made of tungsten, provided by Hunter Scientific, Essex, UK). Then, the ICM was placed on LN521/E-cadherin matrix in a 20 µl drop of TESR2 (supplemented with 20 mg/ml of recombinant human albumin) or NutriStem hESC XF medium, under oil. After the derivation, the cells of new lines were passaged mechanically several times. After the adaptation period, the cells were passaged using the standard procedure of this study, i.e. in single cell suspensions.

Derivation of new hES cell lines from single blastomeres

The work was done in accordance with ethics approval issued to us by the Regional Ethics Board in Stockholm. The donated cleavage stage embryos, frozen two days after *in vitro* fertilization, were thawed and cultured for one more day. Single blastomeres were biopsied in

droplets of 30 µl biopsy medium G-PGD (Vitrolife) under mineral oil. For a biopsy, the embryo was fixed by a holding pipette, and the zona pellucida (ZP) was pierced gently using a Saturn laser (Research Instruments, Ltd, Cornwall, United Kingdom). Once blastomere was acquired, immediately transferred to droplets containing fresh culture medium to be rinsed one time and, after that, placed on LN-521/E-cadherin matrix in a 20 µl drop of medium under oil.

SNP 6.0 array

Copy number variations (CNVs) and copy-number neutral loss-of-heterozygosity (LOH) regions were analyzed using Affymetrix Genome-Wide Human SNP Array 6.0 array and Genotyping Console Software version 4.1 (Affymetrix). The analysis was done using default parameters and comparing each sample against a reference set of 75 healthy individuals generated earlier in the same lab for a different project. The minimum size of CNV segments was 5 markers and 50 kb. Segments with 100% overlap with known CNV regions (according to the Toronto Database of Genomic Variants) were removed from the depicted results.

Statistics

Statistical significance was determined the by Student's two-tailed t-test for unequal variances.

Paper III

Clinical outcome measures

Blood was collected as standard practice during routine care in the intensive care unit. Bronchoalveolar lavage (BAL) fluid was obtained from lavage performed at intervals as part of the routine clinical care provided to each patient. Blood samples for cell purification were collected in heparinized tubes. Serum and plasma samples for cytokines and extracellular vesicles were collected and frozen immediately after sample preparation and stored in -80 ºC until analyzed. Chest X-rays (CXRs) and chest computerized tomography (CT) scans were done as part of the routine clinical care provided to each patient. Respiratory measurements including lung volumes and pressure were obtained from the mechanical ventilation record for each patient. Other standard laboratory measurements were made as part of routine care for each patient and obtained from the patient's record.

BAL Fluid and serum analyses

Levels of pro- and anti-inflammatory cytokines in serial samples of BAL fluid and serum were assessed using a multiplex cytokine assay (Millipore) on a Luminex machine (Millipore). In BAL fluid, surfactant protein B concentration was determined by enzyme-linked immunosorbant assay (ELISA) (Uscn Life Science Inc). Caspase-cleaved K18 (ccK18) and total K18 were measured using M30-Apoptosense® ELISA (Peviva AB) and M65 EpiDeath® ELISA (Peviva AB), respectively.

Assessment of circulating miRNAs

Inhibitory miRNAs were isolated from circulating extracellular vesicles (EVs) purified from the routine blood samples collected. EV purification and miRNA isolation were performed by Exosome Diagnostic Inc. In brief, 1.5-2 ml of blood sample from each time point was purified using the exoRNeasy serum/plasma kit (Qiagen). The eluted RNA was processed for microRNA analysis using the Low Sample Input protocol for the TaqMan® OpenArray®

Human MicroRNA Panel (Life Technologies). Megaplex™ RT Primers, Human Pool A and Human Pool B were used for reverse transcription followed by a pre-amplification step according to manufacturer's protocol. Samples were loaded onto OpenArray® plates using the standard Accufill™ protocol. Amplification was performed according to the protocol established for the TaqMan® OpenArray® Human MicroRNA panel downloaded for each plate (OpenArray Plate product page at www.lifetechnologies.com). In total 758 miRNAs were assayed and 200 to 300 miRNAs gave a detectable CT value for each time point. U6 snRNA (non-coding small nuclear RNA) was used for normalization of miRNA.

Mononuclear cell collection, cell purification, and *In Vitro* mixed lymphocyte studies

Peripheral blood mononuclear cells (PBMCs) used in *in vitro* studies were retrieved from the patients and from healthy donors, isolated by density gradient-based centrifugation, and stored in 10% DMSO in liquid nitrogen until further analysis. For further purification of T-cells, a paramagnetic bead-based selection was utilized (Miltenyi Biotec). The same BM-MSCs as utilized in the patient were co-cultured with allogeneic T-cells. T-cells were stimulated with activating anti-CD2/CD3/CD28 antibodies (Miltenyi Biotec). PBMCs were cultured for five days in the presence of MSCs and the monocytic compartment was subsequently analyzed. Polymorphonuclear leukocytes (PMNs) from buffy coats of healthy donors were ultra-purified under endotoxin-free conditions. PMNs (> 95% purity) and MSCs were co-cultured for up to 40h in presence or absence of LPS (Invitrogen). In all cases, MSCs were plated 72 hours before the start of co-cultures. In selected experiments, MSCs were pre-treated with recombinant human IFN-γ (Peprotech Rocky Hill), and TNF-α (R&D Systems), and finally co-cultured with freshly isolated PMNs. MSC and PMN co-cultures were stained with May Grünwald-Giemsa dye to observe cell morphology following reciprocal interaction.

In Vitro Polymorphonucelar Cell (PMN) Assessments

PMNs were stained according to the manufacturer's instruction with fluorochrome-coupled antibodies. The LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Life technologies) was

used for the exclusion of dead cells for PBMC analysis, and propidium iodide (PI) (Invitrogen) staining for testing the viability of MSCs. For performing intracellular staining, cells were treated with the BD Cytofix/Cytoperm Fixation/Permeabilisation Kit (BD Biosciences). After co-culture with MSCs, PMNs were identified on the basis of their typical morphological parameters (forward scatter/side scatter) and their CD45 expression. An Annexin-V-FITC staining kit (Miltenyi Biotec) was used to assess the levels of PMN apoptosis. Cells were analyzed using a FACS Canto II cytometer (BD Biosciences) and FlowJo Version 9·5 software (TreeStar).

RNA preparation and quantitative RT-PCR

Total RNA was extracted (RNeasy mini kit; Qiagen) and cDNA prepared (Superscript First Strand Synthesis System, Life Technologies) using a Mastercycler nexus (Eppendorf). For cDNA transcription a two-step procedure was performed in line with the manufacturer's recommendations. Messenger RNA levels were quantified by qPCR (Quantitect SYBR Green PCR Kit; Qiagen) on a Rotor Gene Q (Qiagen). Relative gene expression was determined by normalizing to the expression of beta2-microglobulin gene.

In Vitro MSC responses to inflammatory stimuli

The MSCs were tested *in vitro* for their responsiveness towards inflammatory stimuli ("MSC licensing"). Culture in the presence of TNF-α (15 ng/mL) or IFN-γ (10 ng/mL) for 48h induced the expression of CD54 (ICAM-1), CD106 (VCAM-1) and HLA-ABC and –DR, as assessed by flow cytometry, and indoleamine-2,3-dioxygenase (IDO) mRNA transcripts was measured by quantitative PCR .

MSC EV purification

MSCs were cultured in serum-reduced medium (OptiMem, Gibco) for 48 hours. The conditioned medium was harvested and spun and filtered through 0.2 μm sterile syringe filters to remove cell debris. The EVs were subsequently pelleted by ultracentrifugation. The

pellets were re-suspended in PBS, pooled and ultra-centrifuged. The remaining pellet was re-suspended in PBS. Nanoparticle tracking analysis (NTA) was performed using the NS500 (NanoSight, United Kingdom) to measure the size distribution of particles, which is based on the motion and light-scatter of nanometer-sized particles (Brownian motion). The number of particles and their movement were recorded using a camera level of 15 and automatic functions for all post-acquisition settings except for the detection threshold, which was fixed at 6. The samples were diluted in PBS between 1:500 to 1:2,000 to achieve a particle count of between 2 x 10^8 and 2 x 10^9 per ml. The camera focus was adjusted to make the particles appear as sharp dots. Using the script control function, 5x30 seconds videos were recorded; incorporating a sample advance and a 5 seconds delay between each recording and analyzed using the NS500 software. The EVs were frozen at -80 C until further analysis.

EV preparation for proteomic assessment

Donor cell pellet and EVs were lysed with 4% SDS, 25 mM HEPES, 1 mM DTT. Lysates were heated to 95°C followed by sonication and centrifugation. The supernatant was mixed with 1 mM DTT, 8 M urea, 25 mM HEPES, pH 7.6 and transferred to a centrifugation filtering unit, 10 kDa cutoff (Pall, Nanosep®), and centrifuged, followed by another addition of the 8 M urea buffer and centrifugation. Proteins were alkylated by 50 mM Indole-3-acetic acid, in 8 M urea, 25 mM HEPES for 10 min, centrifuged, followed by 2 more additions and centrifugations with 8 M urea, 25 mM HEPES. Trypsin (Promega), 1:50, trypsin:protein, was added to the cell lysate in 250 mM urea, 50 mM HEPES and incubated overnight at 37 °C. The filter units were centrifuged, followed by another centrifugation with MilliQ water and the flow-through was collected. Peptides were cleaned by a strata-X-C-cartridge (Phenomenex).

NanoLC-MS/MS analysis

Before analysis of the Q Exactive mass spectrometer (Thermo Fisher Scientific), peptides were separated using an Agilent 1200 nano-LC system. Samples were trapped on a Zorbax

300SB-C18, and separated on a NTCC-360/100-5-153 (Nikkyo Technos., Ltd) column using a gradient of A (5% DMSO, 0.1% Formic Acid) and B (90% Acetonitrile, 5% DMSO, 0.1% FA), ranging from 5 % to 37 % B in 240 min with a flow of 0.4 µl/min. The Q Exactive MS was operated in a data dependent manner, selecting top 5 precursors for fragmentation by Higher Energy C-trap Dissociation (HCD). The survey scan was performed at 70,000 resolutions from 300-1700 *m/z,* with a max injection time of 100 ms and target of 1 x 10^6 ions. For generation of HCD fragmentation spectra, a max ion injection time of 500 ms and AGC of 1 x 10^5 were used before fragmentation at 30% normalized collision energy, 35,000 resolutions. Precursors were isolated with a width of 2 *m/z* and put on the exclusion list for 70 s. Single and unassigned charge states were rejected from precursor selection.

Peptide, protein identification and data Analyses

Proteome discoverer 1.4 with sequest-percolator was used for protein identification. Precursor mass tolerance was set to 10 ppm and for fragments to 0.02 Da. Oxidized methionine was set as dynamic modification, and carbamidomethylation as static modification. Spectra were matched to a combined database of Uniprot human (140203) combined with the 250 most abundant proteins from 4h nLC-MS/MS analysis of FBS (*Bos taurus*, uniprot 140203), and results were filtered to 1% FDR. Identifications in bos taurus was considered to originate from FBS and removed. GO term enrichment analysis was done using Panther (115).

Statistical Analyses

Wilcoxon paired test was used to compare the differences between two different groups. One-way ANOVA analysis was used to statistically evaluate the difference of sample means among multiple groups. Significant level was set at a p-value ≤ 0.05.

3 AIM, RESULTS AND DISCUSSION

Aim

The general aim of this thesis was to develop gene and cell therapy methods for the use in regenerative medicine. Paper I and II are preclinical work investigating fundamental aspects of gene transfer and cell culturing methodology that could be implemented into clinical protocols in the future. Paper III is a case report describing translational cell treatment in patients with ARDS.

Specific aims.

Paper I. To study the *in vivo* and *in vitro* properties of a novel peptide conjugate and elaborate on mechanisms for improved uptake.

Paper II To produce a protocol for xeno-free derivation of human embryonic stem cells.

Paper III To describe the clinical course in patients with severe ARDS on ECMO and to correlate the clinical outcome to *in vitro* mechanistic parameters.

Paper I: A Peptide-based Vector for Efficient Gene Transfer *In Vitro* and *In Vivo*

In this paper we investigated the use of stearyl-TP10 peptide for delivery of plasmid DNA in cell culture and in mice using intra muscular and intra dermal delivery. The aim was to combined a functional cell penetrating peptide, TP10, with stearyl to enhance endosomal escape. Endosomal escape has been a bottleneck for plasmid delivery and several methods has been described to overcome this problem (116, 117). By adding the stearyl moiety to the TP10 peptide we could increase the expression of plasmids in cell culture up to 4 logs in comparison with naked plasmid delivery (Fig. 2b 3a, b, d and 4b). The mechanism has not fully been investigated, but the increased expression could be attributed to effective condensation (Fig 1b, Table 1) resulting in protection against degradation (Fig 1c-e). Fluorescently labeled plasmid uptake was equal between TP10 and Stearyl-TP10 groups (Fig 2e) in absence of serum and the expression of the TP10/plasmid could not be enhanced by the endosomolytic agent chloroquine (Fig 2a). This data supports that endosomal escape might not be the predominant mechanism. One hypothesis could be that the lack of effect of TP10/plasmid could be rapid dissociation of peptides from plasmids in presence of serum or the endosomal environment prohibiting either cell entry or yielding degradation in the endosome. This argument is supported by TP10 inability to promote plasmid uptake in presence of serum and that heparin dissociates the TP10/plasmid complexes showing that these complexes are very labile (Figures 2f and 1e). Addition of forms more stable particles with plasmids and protecting the DNA from degradation.

We could also demonstrate charge dependent as well as dose dependent intra muscular and intra dermal uptake in mice (Fig 5 a-d). The increase in plasmid expression was only seen in

with charge ratio 1:1 plasmid:TP10-stearyl. These findings show the importance of condensation of plasmid for *in vivo* delivery. Too low charge ratios do not improve on plasmid transfection *in vivo*. Too high charge ratios yield condensed nanoparticles hampering either uptake or expression in the nucleus of the plasmid. These results are corroborated by the inability of heparin to displace peptide from the nanoparticle at high charge ratios. Since transfection efficacy increased with higher charge ratios *in vitro*, the main cause could be due to different mechanisms of cellular uptake *in vitro* and *in vivo* rather than intracellular events. These findings of the relevance of charge ratio for efficient *in vivo* gene transfer and the poor correlations of *in vitro* and *in vivo* results have been discussed in other publications (118, 119).

This study has several limitations. There is no control for TP10/plasmid nano particle *in vivo*. The reasoning was to only test the favorable constructs in the *in vitro* assays *in vivo*. But, as explained above, since *in vitro* data can correlate poorly to the *in vivo* data this control could have been valuable. Furthermore there are only basic toxicity assessments and this should be developed before possible clinical use. Although not the aim of the paper, the use of stearyl-TP10 as a transfection agent for siRNA could be beneficial, since the basic mechanism is to promote endosomal escape. The use of stearyl-TP10 peptide in combination with siRNA has since been demonstrated in model system for cochlear cultures (120) .

Paper II: Clonal culturing of human embryonic stem cells on laminin-521/E-cadherin matrix in defined and xeno-free environment

In this paper we investigated natural conditions that allow clonal survival of hPSCs and derivation of new hESC lines under xeno-free and chemically defined conditions. As described above, laminins are important class of extracellular matrix (ECM) proteins. All the laminin isoforms that are expressed in hPSCs were produced as recombinant proteins, used as

cell culture substrata and were tested with regard to their ability to support self-renewal of hPSCs. Only LN-521 and LN-511, which had been previously shown to facilitate self-renewal of hPSCs (59), allowed attachment and proliferation of hPSCs. Laminin-521, unlike LN-511, also allowed efficient survival and proliferation of individual hPSCs plated at densities higher than 5 000 cells per cm^2. Time-lapse imaging of living cells and analysis of actin-myosin rearrangements demonstrated that LN-521 induced higher motility of hPSCs in comparison with other tested cell culture substrata and that the cells on LN-521 survived through fast migration to each other. Culturing of hPSCs on LN-521 provided much faster multiplication of hPSC number with time than that on other tested ECM proteins. The easy and robust methods described in the paper allow efficient self-renewal of hPSCs under xeno-free and chemically defined conditions and may be useful for development of cell lineages aimed at clinical application in the future.

Individualized hPSCs plated at cloning density (less than 1 000 cells per cm^2) die from anoikis (121) that is a form of apoptosis. Cadherin and ECM mediated signaling is capable to prevent anoikis. In the paper we tested cell adhesion substrata composed of various ECM molecules and E-cadherin that is abundantly expressed on the hPSC membrane. A mixture of LN-521 and E-cadherin (taken at 9:1 w/w ratio) allowed efficient clonal survival of hPSCs. Cloning of hPSCs may be useful for many scientific and medical applications, e.g. it may facilitate manipulation of the human iPSC genomes to repair genetic mutations.

Although many methods allowing self-renewal of hPSCs under xeno-free and chemically defined conditions have been published to date (59-61, 122), no such methods for derivation of new hESC lines have been reported until very recently. This paper described an efficient procedure that facilitated derivation of new hESC lines under fully xeno-free and chemically defined conditions both from the inner cell masses of blastocysts and from single blastomeres. The latter may allow derivation of new hESC lines without destruction of a

parental embryo addressing an important ethical concern of many. Human ESCs are already used in medicine and still considered as "gold standard" pluripotent cells (40). Free from the ethical dilemma and developed in fully defined environment hESCs may significantly facilitate clinical treatments involving them.

Paper III *In vivo* effects of mesenchymal stromal cells in two patients with severe acute respiratory distress syndrome

This paper describes the use of allogeneic transplantation of mesenchymal stromal cells in two patients with severe ARDS on ECMO support. Since this is a small case series the therapeutic benefit can only be speculated. Progressive clinical improvement could be seen shortly after the infusion of the cells. Improvement of lung function in both patients was demonstrated by increased compliance, tidal volume as well as chest X-rays. The clinical improvement continued and both patients were successfully weaned off ECMO (Fig 1 and 2). The paper also demonstrates the feasibility of administration of MSCs into the right atrium through a venous cannula during ECMO support. Apart from pneumonia in patient 1 that could be due to MSC treatment no advert events were seen. The range of improvements in the *in vivo* inflammatory indices was broad. Decreases in multiple markers of inflammation, including markers of epithelial apoptosis, and proinflammatory cytokines, miRNAs, and chemokines in plasma and BAL fluid could be seen (Fig 3 a-e). Although the relevance of specific molecules are not clear in ARDS they could play an important role for restoring an energy deficit in the ARDS-injured lung, and restoring normal surfactant production. Another interesting finding was that the the nosocomial pneumonia in patient 1 could be detected not only by traditional markers but also by miRNA profiles in the extracellular vesicles (3e). This is another interesting field were one can use miRNA in diagnostics of inflammation. The paper also tries to outline assays to correlate *in vivo* to *in vitro* data. This is exemplified in

Figure I-J, where correlation could be seen in MSCs' ability of Treg induction, which could be observed both *in vitro* and *in vivo*. Although Treg induction is slow and cannot explain the rapid improvement in clinical status it demonstrates that a MSC effect correlates well in both settings. In the *in vitro* experiments, we mimicked in part an inflammatory environment by pretreating the ex vivo expanded MSCs with IFN-γ and TNF-α assess MSCs' immune modulatory potency. This can be used as a preclinical batch controls test of MSC before administration. No *in vitro* tests can be used as a perfect biomarker of *in vivo* behavior of MSCs. However, the combination of different *in vitro* standardized tests could possibly be used to increase the correlation of *in vitro* and *in vivo* data. The paper provides a baseline characterization of successfully used MSCs and also their extracellular vesicles. This information could be important for understanding the molecular determinants of functional cell batches of MSCs for clinical use in ARDS. In a similar fashion the experiments showing induction of regulatory phenotypes in monocytes, and neutrophils could if demonstrated consistent in more preclinical as well as in clinical trials to be of value to not only to understand the mechanism of MSC but also to get a deeper knowledge of the ARDS pathology.

The paper has limitations in that this is only a small case series and larger clinical trials are needed to support these findings. Furthermore, the study has no control group so causal improvement can only be speculated upon. These limitations will be addressed in a future clinical study currently on the way. The paper has not penetrated a clear mechanism for the MSCs but many of the therapeutic effects of MSCs in ARDS are likely mediated by the release of soluble proteins and extracellular vesicles that contain a range of bioactive molecules. These molecules have been described in order to develop clinical protocols where more mechanistic questions are addressed. Since the pathology of ARDS is not fully understood it can be difficult at this point to draw to vast conclusions from the mechanistic work in the paper. When it comes to the cytokine, extracellular vesicle, as well as the cellular

data in the paper, it is hypothesis generating and need to be further explored in larger numbers of patients. From the clinical point of view, our data contrasts with those observed in a recent study of the administration of non-HLA-matched allogeneic adipose derived MSCs in ARDS patients. Although this study showed short-term improvement in oxygenation after MSC administration, there was no improvement in ventilator-free days, ICU-free days, or length of hospital (123). Another recently published safety study has shown that MSCs are well tolerated and safe but due to small study population no significance in secondary outcome measure was detected. In comparison to expected outcome in the moderate ARDS group, mortality was reduced from 32% to 22% but statistically not significant (114). Difference in outcome could be dependent on cell source, but also due to culture methods. Another possibility is that the differences are related to serendipity due to the small studies presently available.

Concluding remarks and future challenges

Regenerative medicine is a multidisciplinary field that is constantly evolving. Both gene and cell therapies are modalities that can be used in order to achieve regeneration of lost organ function. In both areas there has been a long and cumbersome process for successful implementation into the clinic. Novel medical therapies are usually delayed because of safety issues. Although time consuming, they play a pivotal role in drug development. In retrospect, many early clinical trials that have been carried out prematurely have rather slowed down the development than contributed to clinical implementation. Currently the use of gene therapy in the clinic as standardized therapies is on the way. Also, robust clinical trials using novel cellular therapies are under way. Traditionally gene and cell therapies have been separate research fields but several applications are on the way were the two fields merged.

Gene transfer in combination with stem cell therapy is clearly a treatment for the future (124,

125) . Currently gene transfer vectors for the creation of iPSCs as well as vectors for iPSC and ESC differentiation are hot topics. Vectors are also used for modifying the stem cell or its derived differentiated cell to provide an additional phenotype. An example of this is the use of autologous genetically modified epidermal stem cells transplanted in patients with Junctional Epidermolysis Bullosa (126). Other interesting approaches are the use of bioengineered tissue for transplantation (127). In this emerging field his thesis has outlined technologies that can be combined to the development of new strategies. A modification of the protocol in paper II is currently being assessed for culturing of mesenchymal stromal cells. One of the factors that is important for the efficacy of MSC treatment is the number of passages. The use of matrix molecules as well as soluble factors could potentiate the self-renewal capacity and increase the number of passages with retained immunomodulatory properties. A recent report demonstrated promising results in treating ARDS with interferon-beta-1a (128). The use of genetically modified MSCs with interferon-beta-1a could potentiate the treatment effect. Apart from development of new interesting combinatorial treatments, larger studies are needed to demonstrate robust efficacy of MSCs in the clinic. A phase I/II study is currently on the way in the USA and we are developing a phase I clinical trial with treatment of severe ARDS on ECMO support. Another challenge in cell and gene therapies is to correlate *in vitro* and *in vivo* data. Both papers I and III try to address these problems. There is a substantial need for *in vitro* assays were that predict robust *in vivo* results.

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5 REFERENCES

1. Rogers S. *New Scientist.* 1970:194.
2. Friedmann T, and Roblin R. Gene therapy for human genetic disease? *Science.* 1972;175(4025):949-55.
3. Blaese RM, Culver KW, Miller AD, Carter CS, Fleisher T, Clerici M, Shearer G, Chang L, Chiang Y, Tolstoshev P, et al. T lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years. *Science.* 1995;270(5235):475-80.
4. Raper SE, Chirmule N, Lee FS, Wivel NA, Bagg A, Gao GP, Wilson JM, and Batshaw ML. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol Genet Metab.* 2003;80(1-2):148-58.
5. Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, Gross F, Yvon E, Nusbaum P, Selz F, Hue C, Certain S, Casanova JL, et al. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science.* 2000;288(5466):669-72.
6. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P, Lim A, Osborne CS, Pawliuk R, Morillon E, et al. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science.* 2003;302(5644):415-9.
7. Gaspar HB, Cooray S, Gilmour KC, Parsley KL, Zhang F, Adams S, Bjorkegren E, Bayford J, Brown L, Davies EG, et al. Hematopoietic stem cell gene therapy for adenosine deaminase-deficient severe combined immunodeficiency leads to long-term immunological recovery and metabolic correction. *Sci Transl Med.* 2011;3(97):97ra80.
8. Ye W, Liu R, Pan C, Jiang W, Zhang L, Guan Z, Wu J, Ying X, Li L, Li S, et al. Multicenter randomized phase 2 clinical trial of a recombinant human endostatin adenovirus in patients with advanced head and neck carcinoma. *Mol Ther.* 2014;22(6):1221-9.
9. Nathwani AC, Reiss UM, Tuddenham EG, Rosales C, Chowdary P, McIntosh J, Della Peruta M, Lheriteau E, Patel N, Raj D, et al. Long-term safety and efficacy of factor IX gene therapy in hemophilia B. *N Engl J Med.* 2014;371(21):1994-2004.
10. Williams DA, and Thrasher AJ. Concise review: lessons learned from clinical trials of gene therapy in monogenic immunodeficiency diseases. *Stem Cells Transl Med.* 2014;3(5):636-42.
11. Jacobson SG, Cideciyan AV, Roman AJ, Sumaroka A, Schwartz SB, Heon E, and Hauswirth WW. Improvement and decline in vision with gene therapy in childhood blindness. *N Engl J Med.* 2015;372(20):1920-6.
12. Cavazzana-Calvo M, and Fischer A. Gene therapy for severe combined immunodeficiency: are we there yet? *J Clin Invest.* 2007;117(6):1456-65.
13. Urnov FD, Rebar EJ, Holmes MC, Zhang HS, and Gregory PD. Genome editing with engineered zinc finger nucleases. *Nat Rev Genet.* 2010;11(9):636-46.

14. Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, and Church GM. RNA-guided human genome engineering via Cas9. *Science.* 2013;339(6121):823-6.

15. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, and Mello CC. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature.* 1998;391(6669):806-11.

16. Kennedy S, Rettinger S, Flye MW, and Ponder KP. Experiments in transgenic mice show that hepatocytes are the source for postnatal liver growth and do not stream. *Hepatology.* 1995;22(1):160-8.

17. Thomas CE, Ehrhardt A, and Kay MA. Progress and problems with the use of viral vectors for gene therapy. *Nat Rev Genet.* 2003;4(5):346-58.

18. Sioud M. Overcoming the challenges of siRNA activation of innate immunity: design better therapeutic siRNAs. *Methods Mol Biol.* 2015;1218(301-19.

19. Boutin S, Monteilhet V, Veron P, Leborgne C, Benveniste O, Montus MF, and Masurier C. Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, 5, 6, 8, and 9 in the healthy population: implications for gene therapy using AAV vectors. *Hum Gene Ther.* 2010;21(6):704-12.

20. Erles K, Sebokova P, and Schlehofer JR. Update on the prevalence of serum antibodies (IgG and IgM) to adeno-associated virus (AAV). *J Med Virol.* 1999;59(3):406-11.

21. Manno CS, Pierce GF, Arruda VR, Glader B, Ragni M, Rasko JJ, Ozelo MC, Hoots K, Blatt P, Konkle B, et al. Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat Med.* 2006;12(3):342-7.

22. Baum C, Kustikova O, Modlich U, Li Z, and Fehse B. Mutagenesis and oncogenesis by chromosomal insertion of gene transfer vectors. *Hum Gene Ther.* 2006;17(3):253-63.

23. Bessis N, GarciaCozar FJ, and Boissier MC. Immune responses to gene therapy vectors: influence on vector function and effector mechanisms. *Gene Ther.* 2004;11 Suppl 1(S10-7.

24. Bouard D, Alazard-Dany D, and Cosset FL. Viral vectors: from virology to transgene expression. *Br J Pharmacol.* 2009;157(2):153-65.

25. Pack DW, Hoffman AS, Pun S, and Stayton PS. Design and development of polymers for gene delivery. *Nat Rev Drug Discov.* 2005;4(7):581-93.

26. Mintzer MA, and Simanek EE. Nonviral vectors for gene delivery. *Chem Rev.* 2009;109(2):259-302.

27. Lehto T, Kurrikoff K, and Langel U. Cell-penetrating peptides for the delivery of nucleic acids. *Expert Opin Drug Deliv.* 2012;9(7):823-36.

28. Mae M, Andaloussi SE, Lehto T, and Langel U. Chemically modified cell-penetrating peptides for the delivery of nucleic acids. *Expert Opin Drug Deliv.* 2009;6(11):1195-205.

29. Pae J, and Pooga M. Peptide-mediated delivery: an overview of pathways for efficient internalization. *Ther Deliv.* 2014;5(11):1203-22.

30. Nakamura Y, Kogure K, Futaki S, and Harashima H. Octaarginine-modified multifunctional envelope-type nano device for siRNA. *J Control Release.* 2007;119(3):360-7.

31. Futaki S, Ohashi W, Suzuki T, Niwa M, Tanaka S, Ueda K, Harashima H, and Sugiura Y. Stearylated arginine-rich peptides: a new class of transfection systems. *Bioconjug Chem.* 2001;12(6):1005-11.

32. Khalil IA, Futaki S, Niwa M, Baba Y, Kaji N, Kamiya H, and Harashima H. Mechanism of improved gene transfer by the N-terminal stearylation of octaarginine: enhanced cellular association by hydrophobic core formation. *Gene Ther.* 2004;11(7):636-44.

33. Lehto T, Abes R, Oskolkov N, Suhorutsenko J, Copolovici DM, Mager I, Viola JR, Simonson OE, Ezzat K, Guterstam P, et al. Delivery of nucleic acids with a stearylated (RxR)4 peptide using a non-covalent co-incubation strategy. *J Control Release.* 2010;141(1):42-51.

34. Bradley JA, Bolton EM, and Pedersen RA. Stem cell medicine encounters the immune system. *Nat Rev Immunol.* 2002;2(11):859-71.

35. Mathe G, Amiel JL, Schwarzenberg L, Cattan A, and Schneider M. Haematopoietic Chimera in Man after Allogenic (Homologous) Bone-Marrow Transplantation. (Control of the Secondary Syndrome. Specific Tolerance Due to the Chimerism). *Br Med J.* 1963;2(5373):1633-5.

36. Thomas ED, Lochte HL, Jr., Lu WC, and Ferrebee JW. Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy. *N Engl J Med.* 1957;257(11):491-6.

37. Le Blanc K, Rasmusson I, Sundberg B, Gotherstrom C, Hassan M, Uzunel M, and Ringden O. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet.* 2004;363(9419):1439-41.

38. Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, Lanino E, Sundberg B, Bernardo ME, Remberger M, et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet.* 2008;371(9624):1579-86.

39. Schwartz SD, Regillo CD, Lam BL, Eliott D, Rosenfeld PJ, Gregori NZ, Hubschman JP, Davis JL, Heilwell G, Spirn M, et al. Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies. *Lancet.* 2014.

40. Simonson OE, Domogatskaya A, Volchkov P, and Rodin S. The safety of human pluripotent stem cells in clinical treatment. *Ann Med.* 2015;47(5):370-80.

41. Domogatskaya A, Rodin S, and Tryggvason K. Functional diversity of laminins. *Annu Rev Cell Dev Biol.* 2012;28(523-53.

42. Aumailley M, Bruckner-Tuderman L, Carter WG, Deutzmann R, Edgar D, Ekblom P, Engel J, Engvall E, Hohenester E, Jones JC, et al. A simplified laminin nomenclature. *Matrix Biol.* 2005;24(5):326-32.

43. Yurchenco PD. Basement membranes: cell scaffoldings and signaling platforms. *Cold Spring Harb Perspect Biol.* 2011;3(2).

44. Talts JF, Sasaki T, Miosge N, Gohring W, Mann K, Mayne R, and Timpl R. Structural and functional analysis of the recombinant G domain of the laminin alpha4 chain and its proteolytic processing in tissues. *J Biol Chem.* 2000;275(45):35192-9.

45. Smirnov SP, McDearmon EL, Li S, Ervasti JM, Tryggvason K, and Yurchenco PD. Contributions of the LG modules and furin processing to laminin-2 functions. *J Biol Chem.* 2002;277(21):18928-37.

46. Yu H, and Talts JF. Beta1 integrin and alpha-dystroglycan binding sites are localized to different laminin-G-domain-like (LG) modules within the laminin alpha5 chain G domain. *Biochem J.* 2003;371(Pt 2):289-99.

47. Talts JF, Andac Z, Gohring W, Brancaccio A, and Timpl R. Binding of the G domains of laminin alpha1 and alpha2 chains and perlecan to heparin, sulfatides, alpha-dystroglycan and several extracellular matrix proteins. *Embo J.* 1999;18(4):863-70.

48. El Nemer W, Gane P, Colin Y, Bony V, Rahuel C, Galacteros F, Cartron JP, and Le Van Kim C. The Lutheran blood group glycoproteins, the erythroid receptors for laminin, are adhesion molecules. *J Biol Chem.* 1998;273(27):16686-93.

49. Udani M, Zen Q, Cottman M, Leonard N, Jefferson S, Daymont C, Truskey G, and Telen MJ. Basal cell adhesion molecule/lutheran protein. The receptor critical for sickle cell adhesion to laminin. *J Clin Invest.* 1998;101(11):2550-8.

50. Roberts DD, Rao CN, Magnani JL, Spitalnik SL, Liotta LA, and Ginsburg V. Laminin binds specifically to sulfated glycolipids. *Proc Natl Acad Sci U S A.* 1985;82(5):1306-10.

51. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, and Jones JM. Embryonic stem cell lines derived from human blastocysts. *Science.* 1998;282(5391):1145-7.

52. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, and Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell.* 2007;131(5):861-72.

53. Revazova ES, Turovets NA, Kochetkova OD, Kindarova LB, Kuzmichev LN, Janus JD, and Pryzhkova MV. Patient-specific stem cell lines derived from human parthenogenetic blastocysts. *Cloning Stem Cells.* 2007;9(3):432-49.

54. Tachibana M, Amato P, Sparman M, Gutierrez NM, Tippner-Hedges R, Ma H, Kang E, Fulati A, Lee HS, Sritanaudomchai H, et al. Human embryonic stem cells derived by somatic cell nuclear transfer. *Cell.* 2013;153(6):1228-38.

55. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, et al. Induced pluripotent stem cell lines derived from human somatic cells. *Science.* 2007;318(5858):1917-20.

56. Hovatta O, Rodin S, Antonsson L, and Tryggvason K. Concise review: animal substance-free human embryonic stem cells aiming at clinical applications. *Stem Cells Transl Med.* 2014;3(11):1269-74.

57. Klimanskaya I, Chung Y, Becker S, Lu SJ, and Lanza R. Human embryonic stem cell lines derived from single blastomeres. *Nature.* 2006;444(7118):481-5.

58. Ludwig TE, Bergendahl V, Levenstein ME, Yu J, Probasco MD, and Thomson JA. Feeder-independent culture of human embryonic stem cells. *Nat Methods.* 2006;3(8):637-46.

59. Rodin S, Domogatskaya A, Strom S, Hansson EM, Chien KR, Inzunza J, Hovatta O, and Tryggvason K. Long-term self-renewal of human pluripotent stem cells on human recombinant laminin-511. *Nat Biotechnol.* 2010;28(6):611-5.

60. Villa-Diaz LG, Nandivada H, Ding J, Nogueira-de-Souza NC, Krebsbach PH, O'Shea KS, Lahann J, and Smith GD. Synthetic polymer coatings for long-term growth of human embryonic stem cells. *Nat Biotechnol.* 2010;28(6):581-3.

61. Melkoumian Z, Weber JL, Weber DM, Fadeev AG, Zhou Y, Dolley-Sonneville P, Yang J, Qiu L, Priest CA, Shogbon C, et al. Synthetic peptide-acrylate surfaces for long-term self-renewal and cardiomyocyte differentiation of human embryonic stem cells. *Nat Biotechnol.* 2010;28(6):606-10.

62. Rodin S, Antonsson L, Niaudet C, Simonson OE, Salmela E, Hansson EM, Domogatskaya A, Xiao Z, Damdimopoulou P, Sheikhi M, et al. Clonal culturing of human embryonic stem cells on laminin-521/E-cadherin matrix in defined and xeno-free environment. *Nat Commun.* 2014;5(3195.

63. Uccelli A, Moretta L, and Pistoia V. Mesenchymal stem cells in health and disease. *Nat Rev Immunol.* 2008;8(9):726-36.

64. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, and Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy.* 2006;8(4):315-7.

65. Tremain N, Korkko J, Ibberson D, Kopen GC, DiGirolamo C, and Phinney DG. MicroSAGE analysis of 2,353 expressed genes in a single cell-derived colony of undifferentiated human mesenchymal stem cells reveals mRNAs of multiple cell lineages. *Stem Cells.* 2001;19(5):408-18.

66. Duijvestein M, Vos AC, Roelofs H, Wildenberg ME, Wendrich BB, Verspaget HW, Kooy-Winkelaar EM, Koning F, Zwaginga JJ, Fidder HH, et al. Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. *Gut.* 2010;59(12):1662-9.

67. Connick P, Kolappan M, Crawley C, Webber DJ, Patani R, Michell AW, Du MQ, Luan SL, Altmann DR, Thompson AJ, et al. Autologous mesenchymal stem cells for the treatment of secondary progressive multiple sclerosis: an open-label phase 2a proof-of-concept study. *Lancet Neurol.* 2012;11(2):150-6.

68. Simonson OE, Mougiakakos D, Heldring N, Bassi G, Johansson HJ, Dalen M, Jitschin R, Rodin S, Corbascio M, El Andaloussi S, et al. In Vivo Effects of Mesenchymal Stromal Cells in Two Patients With Severe Acute Respiratory Distress Syndrome. *Stem Cells Transl Med.* 2015;4(10):1199-213.

69. Griffin MD, Ryan AE, Alagesan S, Lohan P, Treacy O, and Ritter T. Anti-donor immune responses elicited by allogeneic mesenchymal stem cells: what have we learned so far? *Immunol Cell Biol.* 2013;91(1):40-51.

70. Ankrum J, and Karp JM. Mesenchymal stem cell therapy: Two steps forward, one step back. *Trends Mol Med.* 2010;16(5):203-9.

71. Keating A. Mesenchymal stromal cells: new directions. *Cell Stem Cell.* 2012;10(6):709-16.

72. Heldring N, Mager I, Wood MJ, Le Blanc K, and Andaloussi SE. Therapeutic Potential of Multipotent Mesenchymal Stromal Cells and Their Extracellular Vesicles. *Hum Gene Ther.* 2015;26(8):506-17.

73. Tomchuck SL, Zwezdaryk KJ, Coffelt SB, Waterman RS, Danka ES, and Scandurro AB. Toll-like receptors on human mesenchymal stem cells drive their migration and immunomodulating responses. *Stem Cells.* 2008;26(1):99-107.

74. Prockop DJ, and Oh JY. Mesenchymal stem/stromal cells (MSCs): role as guardians of inflammation. *Mol Ther.* 2012;20(1):14-20.

75. Meisel R, Zibert A, Laryea M, Gobel U, Daubener W, and Dilloo D. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. *Blood.* 2004;103(12):4619-21.

76. Ryan JM, Barry F, Murphy JM, and Mahon BP. Interferon-gamma does not break, but promotes the immunosuppressive capacity of adult human mesenchymal stem cells. *Clin Exp Immunol.* 2007;149(2):353-63.

77. Gieseke F, Schutt B, Viebahn S, Koscielniak E, Friedrich W, Handgretinger R, and Muller I. Human multipotent mesenchymal stromal cells inhibit proliferation of PBMCs independently of IFNgammaR1 signaling and IDO expression. *Blood.* 2007;110(6):2197-200.

78. Yao C, Sakata D, Esaki Y, Li Y, Matsuoka T, Kuroiwa K, Sugimoto Y, and Narumiya S. Prostaglandin E2-EP4 signaling promotes immune inflammation through Th1 cell differentiation and Th17 cell expansion. *Nat Med.* 2009;15(6):633-40.

79. Harizi H. Reciprocal crosstalk between dendritic cells and natural killer cells under the effects of PGE2 in immunity and immunopathology. *Cell Mol Immunol.* 2013;10(3):213-21.

80. Baratelli F, Lin Y, Zhu L, Yang SC, Heuze-Vourc'h N, Zeng G, Reckamp K, Dohadwala M, Sharma S, and Dubinett SM. Prostaglandin E2 induces FOXP3 gene expression and T regulatory cell function in human CD4+ T cells. *J Immunol.* 2005;175(3):1483-90.

81. Carosella ED, Gregori S, Rouas-Freiss N, LeMaoult J, Menier C, and Favier B. The role of HLA-G in immunity and hematopoiesis. *Cell Mol Life Sci.* 2011;68(3):353-68.

82. Carosella ED. The tolerogenic molecule HLA-G. *Immunol Lett.* 2011;138(1):22-4.

83. Nasef A, Mathieu N, Chapel A, Frick J, Francois S, Mazurier C, Boutarfa A, Bouchet S, Gorin NC, Thierry D, et al. Immunosuppressive effects of mesenchymal stem cells: involvement of HLA-G. *Transplantation.* 2007;84(2):231-7.

84. Deschaseaux F, Delgado D, Pistoia V, Giuliani M, Morandi F, and Durrbach A. HLA-G in organ transplantation: towards clinical applications. *Cell Mol Life Sci.* 2011;68(3):397-404.

85. Clark EA, Kalomoiris S, Nolta JA, and Fierro FA. Concise review: MicroRNA function in multipotent mesenchymal stromal cells. *Stem Cells.* 2014;32(5):1074-82.

86. Lai RC, Yeo RW, and Lim SK. Mesenchymal stem cell exosomes. *Semin Cell Dev Biol.* 2015;40(82-8.

87. Akyurekli C, Le Y, Richardson RB, Fergusson D, Tay J, and Allan DS. A systematic review of preclinical studies on the therapeutic potential of mesenchymal stromal cell-derived microvesicles. *Stem Cell Rev.* 2015;11(1):150-60.

88. Gibbon JH, Jr. Application of a mechanical heart and lung apparatus to cardiac surgery. *Minn Med.* 1954;37(3):171-85; passim.

89. Baffes TG, Fridman JL, Bicoff JP, and Whitehill JL. Extracorporeal circulation for support of palliative cardiac surgery in infants. *Ann Thorac Surg.* 1970;10(4):354-63.

90. Bartlett RH, Gazzaniga AB, Jefferies MR, Huxtable RF, Haiduc NJ, and Fong SW. Extracorporeal membrane oxygenation (ECMO) cardiopulmonary support in infancy. *Trans Am Soc Artif Intern Organs.* 1976;22(80-93.

91. Wilson JG, and Matthay MA. Mechanical ventilation in acute hypoxemic respiratory failure: a review of new strategies for the practicing hospitalist. *J Hosp Med.* 2014;9(7):469-75.

92. Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. The Acute Respiratory Distress Syndrome Network. *N Engl J Med.* 2000;342(18):1301-8.

93. Zampieri FG, Mendes PV, Ranzani OT, Taniguchi LU, Pontes Azevedo LC, Vieira Costa EL, and Park M. Extracorporeal membrane oxygenation for severe respiratory failure in adult patients: a systematic review and meta-analysis of current evidence. *J Crit Care.* 2013;28(6):998-1005.

94. Ware LB, and Matthay MA. The acute respiratory distress syndrome. *N Engl J Med.* 2000;342(18):1334-49.

95. Matthay MA, Ware LB, and Zimmerman GA. The acute respiratory distress syndrome. *J Clin Invest.* 2012;122(8):2731-40.

96. Weiss DJ. Concise review: current status of stem cells and regenerative medicine in lung biology and diseases. *Stem Cells.* 2014;32(1):16-25.

97. von Bahr L, Sundberg B, Lonnies L, Sander B, Karbach H, Hagglund H, Ljungman P, Gustafsson B, Karlsson H, Le Blanc K, et al. Long-term complications, immunologic effects, and role of passage for outcome in mesenchymal stromal cell therapy. *Biol Blood Marrow Transplant.* 2012;18(4):557-64.

98. Lalu MM, McIntyre L, Pugliese C, Fergusson D, Winston BW, Marshall JC, Granton J, and Stewart DJ. Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. *PLoS One.* 2012;7(10):e47559.

99. Weiss DJ, Casaburi R, Flannery R, LeRoux-Williams M, and Tashkin DP. A placebo-controlled, randomized trial of mesenchymal stem cells in COPD. *Chest.* 2013;143(6):1590-8.

100. Toonkel RL, Hare JM, Matthay MA, and Glassberg MK. Mesenchymal stem cells and idiopathic pulmonary fibrosis. Potential for clinical testing. *Am J Respir Crit Care Med.* 2013;188(2):133-40.

101. Lee JW, Fang X, Gupta N, Serikov V, and Matthay MA. Allogeneic human mesenchymal stem cells for treatment of E. coli endotoxin-induced acute lung injury

in the ex vivo perfused human lung. *Proc Natl Acad Sci U S A.* 2009;106(38):16357-62.

102. Curley GF, Ansari B, Hayes M, Devaney J, Masterson C, Ryan A, Barry F, O'Brien T, Toole DO, and Laffey JG. Effects of intratracheal mesenchymal stromal cell therapy during recovery and resolution after ventilator-induced lung injury. *Anesthesiology.* 2013;118(4):924-32.

103. Mei SH, McCarter SD, Deng Y, Parker CH, Liles WC, and Stewart DJ. Prevention of LPS-induced acute lung injury in mice by mesenchymal stem cells overexpressing angiopoietin 1. *PLoS Med.* 2007;4(9):e269.

104. Xu J, Qu J, Cao L, Sai Y, Chen C, He L, and Yu L. Mesenchymal stem cell-based angiopoietin-1 gene therapy for acute lung injury induced by lipopolysaccharide in mice. *J Pathol.* 2008;214(4):472-81.

105. Iyer SS, Torres-Gonzalez E, Neujahr DC, Kwon M, Brigham KL, Jones DP, Mora AL, and Rojas M. Effect of bone marrow-derived mesenchymal stem cells on endotoxin-induced oxidation of plasma cysteine and glutathione in mice. *Stem Cells Int.* 2010;2010(868076.

106. Danchuk S, Ylostalo JH, Hossain F, Sorge R, Ramsey A, Bonvillain RW, Lasky JA, Bunnell BA, Welsh DA, Prockop DJ, et al. Human multipotent stromal cells attenuate lipopolysaccharide-induced acute lung injury in mice via secretion of tumor necrosis factor-alpha-induced protein 6. *Stem Cell Res Ther.* 2011;2(3):27.

107. Mei SH, Haitsma JJ, Dos Santos CC, Deng Y, Lai PF, Slutsky AS, Liles WC, and Stewart DJ. Mesenchymal stem cells reduce inflammation while enhancing bacterial clearance and improving survival in sepsis. *Am J Respir Crit Care Med.* 2010;182(8):1047-57.

108. Gupta N, Su X, Popov B, Lee JW, Serikov V, and Matthay MA. Intrapulmonary delivery of bone marrow-derived mesenchymal stem cells improves survival and attenuates endotoxin-induced acute lung injury in mice. *J Immunol.* 2007;179(3):1855-63.

109. Vallabhaneni KC, Haller H, and Dumler I. Vascular smooth muscle cells initiate proliferation of mesenchymal stem cells by mitochondrial transfer via tunneling nanotubes. *Stem Cells Dev.* 2012;21(17):3104-13.

110. Lee JW, Zhu Y, and Matthay MA. Cell-based therapy for acute lung injury: are we there yet? *Anesthesiology.* 2012;116(6):1189-91.

111. Kordelas L, Rebmann V, Ludwig AK, Radtke S, Ruesing J, Doeppner TR, Epple M, Horn PA, Beelen DW, and Giebel B. MSC-derived exosomes: a novel tool to treat therapy-refractory graft-versus-host disease. *Leukemia.* 2014;28(4):970-3.

112. Lai RC, Arslan F, Lee MM, Sze NS, Choo A, Chen TS, Salto-Tellez M, Timmers L, Lee CN, El Oakley RM, et al. Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res.* 2010;4(3):214-22.

113. Zhang Y, Xie RL, Gordon J, LeBlanc K, Stein JL, Lian JB, van Wijnen AJ, and Stein GS. Control of mesenchymal lineage progression by microRNAs targeting skeletal gene regulators Trps1 and Runx2. *J Biol Chem.* 2012;287(26):21926-35.

114. Wilson JG, Liu KD, Zhuo H, Caballero L, McMillan M, Fang X, Cosgrove K, Vojnik R, Calfee CS, Lee JW, et al. Mesenchymal stem (stromal) cells for treatment of ARDS: a phase 1 clinical trial. *Lancet Respir Med.* 2015;3(1):24-32.

115. Mi H, Muruganujan A, and Thomas PD. PANTHER in 2013: modeling the evolution of gene function, and other gene attributes, in the context of phylogenetic trees. *Nucleic acids research.* 2013;41(Database issue):D377-86.

116. Wadia JS, Stan RV, and Dowdy SF. Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat Med.* 2004;10(3):310-5.

117. Erbacher P, Roche AC, Monsigny M, and Midoux P. Putative role of chloroquine in gene transfer into a human hepatoma cell line by DNA/lactosylated polylysine complexes. *Exp Cell Res.* 1996;225(1):186-94.

118. Viola JR, Leijonmarck H, Simonson OE, Oprea, II, Frithiof R, Purhonen P, Moreno PM, Lundin KE, Stromberg R, and Smith CI. Fatty acid-spermine conjugates as DNA carriers for nonviral in vivo gene delivery. *Gene Ther.* 2009;16(12):1429-40.

119. Viola JR, El-Andaloussi S, Oprea, II, and Smith CI. Non-viral nanovectors for gene delivery: factors that govern successful therapeutics. *Expert Opin Drug Deliv.* 2010;7(6):721-35.

120. Dash-Wagh S, Jacob S, Lindberg S, Fridberger A, Langel U, and Ulfendahl M. Intracellular Delivery of Short Interfering RNA in Rat Organ of Corti Using a Cell-penetrating Peptide PepFect6. *Mol Ther Nucleic Acids.* 2012;1(e61.

121. Chen G, Hou Z, Gulbranson DR, and Thomson JA. Actin-myosin contractility is responsible for the reduced viability of dissociated human embryonic stem cells. *Cell Stem Cell.* 2010;7(2):240-8.

122. Miyazaki T, Futaki S, Suemori H, Taniguchi Y, Yamada M, Kawasaki M, Hayashi M, Kumagai H, Nakatsuji N, Sekiguchi K, et al. Laminin E8 fragments support efficient adhesion and expansion of dissociated human pluripotent stem cells. *Nat Commun.* 2012;3(1236.

123. Zheng G, Huang L, Tong H, Shu Q, Hu Y, Ge M, Deng K, Zhang L, Zou B, Cheng B, et al. Treatment of acute respiratory distress syndrome with allogeneic adipose-derived mesenchymal stem cells: a randomized, placebo-controlled pilot study. *Respir Res.* 2014;15(39.

124. Batlevi CL, Matsuki E, Brentjens RJ, and Younes A. Novel immunotherapies in lymphoid malignancies. *Nat Rev Clin Oncol.* 2015.

125. Kalos M, Levine BL, Porter DL, Katz S, Grupp SA, Bagg A, and June CH. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Sci Transl Med.* 2011;3(95):95ra73.

126. De Rosa L, Carulli S, Cocchiarella F, Quaglino D, Enzo E, Franchini E, Giannetti A, De Santis G, Recchia A, Pellegrini G, et al. Long-term stability and safety of transgenic cultured epidermal stem cells in gene therapy of junctional epidermolysis bullosa. *Stem Cell Reports.* 2014;2(1):1-8.

127. Buznyk O, Pasyechnikova N, Islam MM, Iakymenko S, Fagerholm P, and Griffith M. Bioengineered Corneas Grafted as Alternatives to Human Donor Corneas in Three High-Risk Patients. *Clin Transl Sci.* 2015;8(5):558-62.

128. Bellingan G, Maksimow M, Howell DC, Stotz M, Beale R, Beatty M, Walsh T, Binning A, Davidson A, Kuper M, et al. The effect of intravenous interferon-beta-1a (FP-1201) on lung CD73 expression and on acute respiratory distress syndrome mortality: an open-label study. *Lancet Respir Med.* 2014;2(2):98-107.