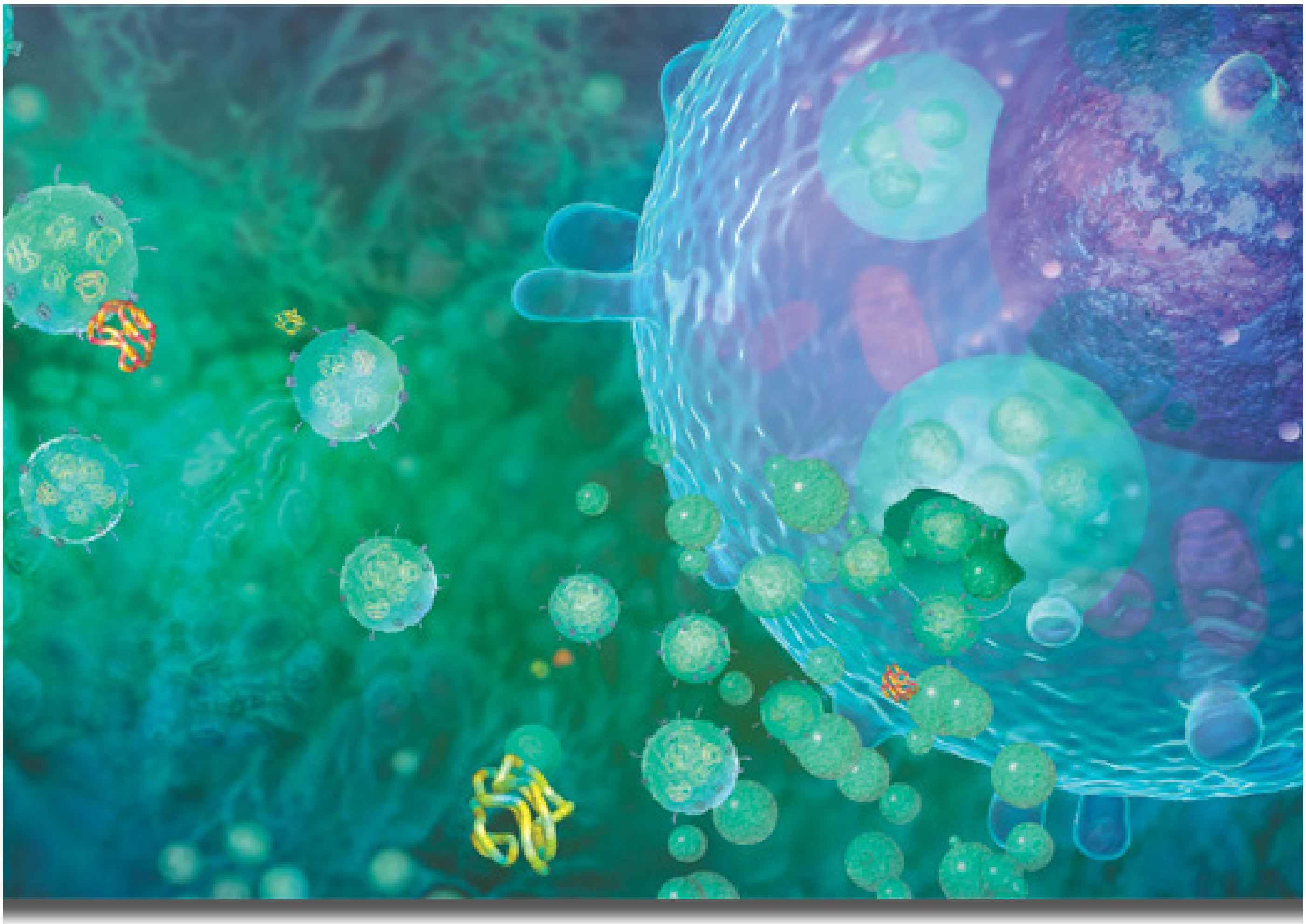

Adipose - Derived Stem Cell Exosomes & Their Relevance In Regenerative Medicine

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May 2014

AD NYE VEJE

AALBORG UNIVERSITET

Medicine with Industrial Specialization
Department of Health Science & Technology
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Magister Scientiae Thesis

Adipose - Derived Stem Cell Exosomes & Their Relevance In Regenerative Medicine

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A thesis submitted in fulfilment of the requirements for the degree of Candidatus Scientiarum Medicinae

in

Medicine with Industrial Specialization
Department of Health Science & Technology

May 2014

Declaration of Authorship

I, Stavros Papaioannou, declare that this thesis titled, ”Adipose–Derived Stem Cell Exosomes & Their Relevance In Regenerative Medicine” and the work presented in it are my own. I confirm that:

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- Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated.
- Where I have consulted the published work of others, this is always clearly attributed.
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"All things are subject to interpretation. Whichever interpretation prevails at a given time is a function of power and not truth."

Friedrich Nietzsche

AALBORG UNIVERSITY

Abstract

Medicine with Industrial Specialization
Department of Health Science & Technology

Candidatus Scientiarium Medicinae

Adipose–Derived Stem Cell Exosomes & Their Relevance In Regenerative Medicine

by Stavros Papaioannou

Adipose-derived stem cells (ASCs) possess potent immunosuppressive and regenerative properties, supposedly mediated by soluble factors and extracellular vesicles (exosomes) secreted by the cells.
In the present study, cell culture standardization and production of particle–depleted serum was of primary importance in order to obtain high quality isolated particles. Using ASCs from three different donors we performed a comprehensive evaluation of current methods used for exosome isolation including ultracentrifugation and polymer–based precipitation (exosome–isolation kit; Invitrogen). We employed hypoxic preconditioning of the aforementioned cell lines in order to assess the effects of low–oxygen–concentration on the production and release of exosomes. The isolated exosomes were assessed by nanoparticle tracking analysis (NTA), visualized by electron and atomic force microscopy (TEM/IEM and AFM) and characterized by extracellular vesicle array (EV array).
The serum–purification protocol adopted in the current study, resulted in the production of virtually particle–free FCS, thus, enabling high standard cell cultures and unbiased downstream EV analysis. All isolated particle fractions, obtained by the isolation methods under investigation, contained 30–100 nm vesicles, as resulted by NTA analysis. However, only kit–isolated samples were positive for exosome markers (CD9, CD63 and CD81) based on electron microscopy and EV array. Polymer–based exosome isolation resulted the most suitable method to isolate secreted vesicles. Cell cultures at 1% oxygen concentration did not display any beneficial morphological or quantitative effect on ASC–derived exosomes, compared to normoxic controls.

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Contents

Declaration of Authorship i

Abstract iii

Acknowledgements iv

List of Figures vii

Abbreviations viii

1 Introduction 1

2 Literature Review 3
- 2.1 Adipose–Derived Stem Cells: Relevance in Regenerative Medicine 3
  - 2.1.1 Biology & Clinical Applications 3
  - 2.1.2 The Immunomodulatory Capacity of the ASCs 5
- 2.2 Extracellular Vesicles 5
  - 2.2.1 Exosomes: Small Vesicles Participating in Cell Communication and Immune Responses 5
  - 2.2.2 ASC–Derived Vesicles as a Novel Approach for Cell–Free Therapy 7
- 2.3 Standardization in ASC–Derived EV Research 8
  - 2.3.1 Bovine Serum–Derived EVs 8
  - 2.3.2 Standardized ASC–Derived Exosome Isolation 9
  - 2.3.3 Hypoxia–Conditioned ASCs 9
  - 2.3.4 Quantitative and Qualitative Evaluation of EVs 10

3 Aim & Objectives 11

4 Materials & Methods 12
- 4.1 Production of Exosome–Free FCS 12
- 4.2 ASC Cultures 12
  - 4.2.1 Relation Between Cell Confluence & Exosome Release 13
- 4.3 Exosome Isolation 13
  - 4.3.1 Polymeric Precipitation 14
  - 4.3.2 Sequential Ultracentrifugation 14

4.4 Protein Quantification (BCA Protein Assay) 15
4.5 Exosome Characterization 15
4.5.1 Nanoparticle Tracking Analysis (NTA) 15
4.5.2 Atomic Force Microscopy (AFM) 16
4.5.3 Transmission Electron Microscopy (TEM) with Immuno–Labeling (IEM) 16
4.5.4 Extracellular Vesicle Array 18
4.6 Statistical Analysis 18

5 Results **19**
5.1 Background 19
5.2 Production of Exosome–Free FCS 19
5.3 Exosome Isolation 21
5.3.1 Polymeric Isolation vs Ultracentrifugation 21
5.4 Oxygen Concentration & Exosome Release 24
5.5 Cell Confluence & Exosome Release 25
5.6 Quantitative & Qualitative Assessment of Exosomes 27
5.6.1 Nanoparticle Particle Analysis 27
5.6.2 Atomic Force Microscopy 27
5.6.3 Electron Microscopy 28
5.6.4 Extracellular Vesicle Array 29

6 Discussion & Conclusions **32**
6.1 Calf Serum–Derived Extracellular Vesicles 32
6.2 Cell Death & Microbial Contamination 33
6.3 Ultracentrifugation & Polymeric Precipitation 33
6.4 Adipose–Derived Stem Cells & Hypoxia 34
6.5 The Effect on Exosomal Yield by Prolonged Culturing Periods 34
6.6 Believe in What You See 35
6.7 Concluding Remarks & Perspectives 36

A AFM High Resolution Images **37**

Bibliography **38**

List of Figures

2.1 Mesenchymal Stem Cells: Origin and Therapeutic Applications 4
2.2 Extracellular Vesicles: classes 6
2.3 Biogenesis of Membrane Vesicles 6
2.4 Exosome–mediated protein and miRNA transfer 7

4.1 Exosome Isolation Workflow and Cell Culture 14
4.2 Extracellular Vesicle Array 18

5.1 Production of Exosome–Free FCS: Schematic Representation 20
5.2 Production of Exosome–Free FCS: Untreated FCS vs Exosome–Free FCS 20
5.3 Production of Exosome–Free FCS: FCS Fractions 21
5.4 Size Distribution Profiles: Kit vs UC Isolated EVs 22
5.5 Total Exosomal Fractions: Kit vs UC 23
5.6 Oxygen Concentration and Exosome Release: Cell Proliferation and Exosome Release 24
5.7 Oxygen Concentration and Exosome Release: Particle Size and Relation Between Exosome Release and Number of Cells 24
5.8 Cell confluence and Exosome Release: ASC 12, 21, 23 and 24 25
5.9 Cell confluence and Exosome Release: Particles released in Relation to Cell Density 26
5.10 Nanoparticle Tracking Analysis 27
5.11 Atomic Force Microscopy 28
5.12 Electron Microscopy 28
5.13 EV Array: Antibodies setup 29
5.14 EV Array: Normoxia vs Hypoxia 30
5.15 EV Array: Kit–Isolated EVs 30
5.16 EV Array: Heatmap 31

A.1 AFM High Res. Images 37

Abbreviations

MSC	**M**esenchymal **S**tem **C**ell
ASC	**A**dipose–derived **S**tem **C**ell
EV	**E**xtracellular **V**esicle
VEGF	**V**ascular **E**ndothelial **G**rowth **F**actor
IL–10	**I**nterleukin–**10**
NO	**N**itrogen **O**xide
TGFβ	**T**ransforming **G**rowth **F**actor beta
CCV	**C**lathrin–**C**oated **V**esicle
MVE	**M**ulti**V**esicular **E**ndosome
STAT–3	**S**ignal **T**ransducer and **A**ctivator of **T**ranscription–3
HIF–1α	**H**ypoxia **I**nducible **F**actor–1α
FCS	**F**etal **C**alf **S**erum
PBS	**P**hosphate **B**uffer **S**aline
NTA	**N**anoparticle **T**racking **A**nalysis
CM	**C**onditioned **M**edium
CCM	**C**oncentrated **C**onditioned **M**edium
BCA	**B**icinchoninic **A**cid
MSD	**M**ean **S**quare **D**isplacement
AFM	**A**tomic **F**orce **M**icroscopy
APTMS	3–**A**mino**P**ropyl **T**rimethoxysilane Tetra**M**ethoxy**S**ilane
TEM	**T**ransmission **E**lectron **M**icroscopy
IEM	**I**mmuno **E**lectron **M**icroscopy
PTA	**P**hospho**T**ungstic **A**cid
mAb	**m**onoclonal **A**nti**b**ody
CD9, 63...	**C**luster of **D**ifferentiation protein 9, 63...

SEM	**S**tandard **E**rror of the **M**ean
UC	**U**ltra**C**entrifugation
EFM	**E**xosome–**F**ree **M**edium
TNF RI	**T**umor **N**ecrosis **F**actor **R**eceptor **I**

To my dedicated wife & wonderful daughter

Chapter 1

Introduction

A great number of medical conditions, such as organ failure, tissue loss due to trauma, cancer ablation or even congenital structural anomalies, can be treated by current clinical procedures including allotransplantation, autologous tissue transfer, and the use of artificial materials; however, these treatment approaches have limitations and risky side effects, including organ shortages, damage to healthy parts of the body during treatment, allergic reactions, and immune rejection.[1]

Recent investigations involving infusion of autologous or allogeneic mesenchymal stem cells (MSCs) have proven successful and the grafts are generally well tolerated.[2, 3] However, new studies also suggest that efficacy may be compromised by lung sequestration and rapid elimination of the transplanted cells. Transient pro–inflammatory effects, opportunistic infections and cancers as well as alloantibody induction are safety issues that might hinder the utilization of MSCs.[4, 5] Animal models indicate that autologous MSCs might reveal efficacious in preventing or treating early intragraft inflammation and may reduce the risk of acute rejection. The potential for donor–specific allogeneic MSCs to promote allograft tolerance is suggested by animal model studies but has not yet been proven in humans.[6]

While tissue engineering and regenerative medicine are undertaking the quest of finding the most suitable type of stem cells that could be employed for therapy, various types of more differentiated adult stem and progenitor cells are in meantime being employed in various clinical trials to replace or regenerate damaged organs.[7, 8] It is noteworthy that, for a great variety of the applied stem cells, the currently observed final outcomes of cellular therapies are often similar. This fact and the lack of convincing evidence for donor–recipient successful chimerism in treated tissues in most of the studies indicates that the transdifferentiation of cells infused systemically into peripheral blood or injected directly into damaged organs may not be the main mechanism involved.[9]

The wide experimental effort put into understanding the mechanisms underlying communication between cells and immune system lead to the characterization and comprehension of secreted membrane vesicles. Although, there is not still a consensus regarding their classification and nomenclature, big effort is being put into isolating their various fractions and using them in several therapeutic procedures, such as treating acute kidney injury, controlling graft versus host disease and curing autoimmune diseases.[10–12]
Even though, the mechanisms underlying the function of microvesicles and exosomes are still under intense investigation, it has been shown that they are able to affect the physiology of neighboring cells in various ways, from inducing intracellular cascade signaling upon receptor binding to conferring new properties after the acquisition of enzymes, new receptors or even genetic material.[13, 14]
Current data and ongoing investigations suggest that ASCs may not only replace damaged or diseased tissues, but also exert several trophic, regenerative and anti–inflammatory effects by either paracrine or endocrine means. However, in order to fully comprehend the properties of ASC–derived EVs, technical standardization is of central importance, as numerous methodologies have been employed to isolate and assess secreted vesicles. The influence of these diverse techniques on downstream EV–quantification and phenotypization remains unclear, raising the need to provide a clear definition of "best practices" and standardization.[15–17]
The disparity of the results, dependent, in part, on procedural differences in EV–research area, leads to the aim of the current work, which is to define the ideal ASC–culturing methods and particle–recovery techniques as well as to investigate their reproducibility throughout the day.

Chapter 2

Literature Review

2.1 Adipose–Derived Stem Cells: Relevance in Regenerative Medicine

2.1.1 Biology & Clinical Applications

A growing number of investigations suggest that human adipose–derived stem cells (ASCs) possess high developmental plasticity both *in vivo* and *in vitro*, and might be a possible candidate to a viable cell source for therapeutic angiogenesis, tissue engineering and cell therapy.[18, 19]
Human ASCs can be easily harvested in big quantities using minimally invasive techniques and they can be expanded *in vitro*.[20] Acclaimed and well documented regenerative features of ASCs include secretion of restorative trophic factors, multilineage differentiation capacity, immunosuppression of activated immune cells and homing to areas of injury.[21–23] These are characteristics that ASCs share with the well characterized bone marrow–derived stem cells (MSCs) (Fig. 2.1).[24–27]

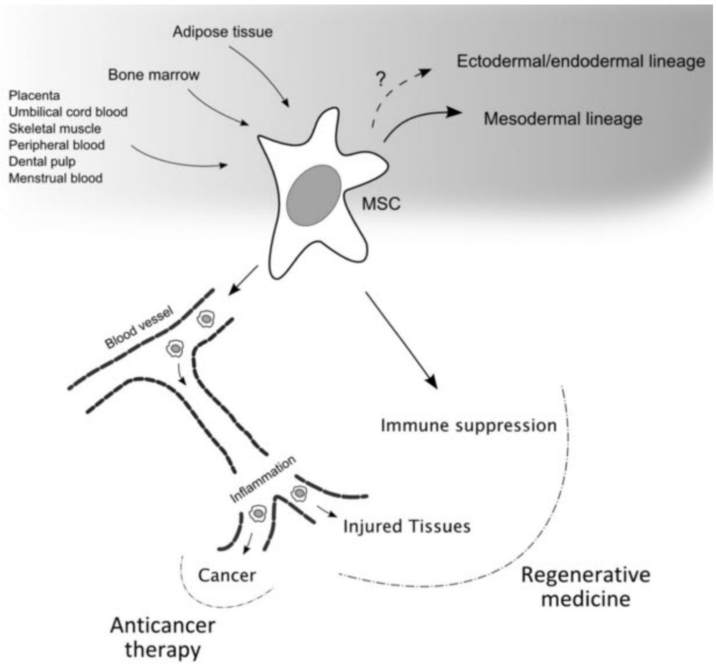

FIGURE 2.1: Mesenchymal stem cells (MSCs) can be isolated from different sources, in particular from bone marrow and adipose tissues, and can differentiate into various cell types of the mesodermal germ layer. Two main properties make MSCs good candidates for therapeutic applications. First, their ability to enter the blood circulation and home to sites of inflammation, i.e., damaged and cancerous tissues, where MSCs can release a multitude of trophic factors. Second, MSCs have the ability to suppress the immune system via different mechanism. Abilities exerted by the MSCs that can be exploited in anticancer therapy and regenerative medicine. Modified from [28]

The multilineage differentiation ability of ASCs can be, among other clinical applications, harnessed in orthopedic applications and directly contribute to repairing damaged tissue through *de novo* cartilage or bone formation.[29] ASCs express and release a range of different growth factors, including key potent regulators of angiogenesis as VEGF. This property may have substantial clinical value, for example, treating peripheral vascular diseases or enhancing wound healing.[30]

Tissue–engineered ASCs have been employed, with success, in reconstructive surgery for patients who received partial mastectomy for breast cancer, by using a combination of autologous adipose tissue and concentrated autologous adipose tissue–derived regenerative cells. Intense ongoing investigations, focus on administrating stem cells, at early stages, in patients who suffered myocardial infarction to hopefully reduce scarring of the myocardium and thus improve myocardial performance.[31–36]

2.1.2 The Immunomodulatory Capacity of the ASCs

ASCs exhibit in vitro immunosuppressive properties with therapeutic potential to prevent graft–versus–host disease in allogeneic hematopoietic cell transplantation, enhance the wound healing process, reduce and improve the foreign body response in the use of biomaterials for therapeutic purposes.[37–40]
Whether the immunomodulatory capacity of ASCs is due to their developmental plasticity, the composition of their secreted fraction or other factors is under intense investigation. [41, 42] In addition to their differentiation potential, ASCs exhibit pleiotropic immune regulatory activities, which are mediated by complex mechanisms. These include cell–cell contact and release of soluble factors such as IL-10, VEGF, NO, TGFβ and many more, which produce diverse effects on the different immune cell subpopulations of the innate and adaptive immunity. Inhibition of proliferation, cytotoxicity, regulation of migration, inhibition of apoptosis, promotion of chemokine production, release of growth factors and other immunosuppressive factors are some of the functions identified by the ASCs.[19, 43–46]
Comprehending the mechanisms behind ASCs immunomodulatory capacity is of vital importance in order to exploit their therapeutic potential. As mentioned above, the release of soluble factors by the ASCs and their immunomodulatory potential are well documented and under intense investigation, but another mode of intercellular communication –the release of membrane vesicles– with important implications in modulating the immune response, has recently become the subject of increasing interest.[10]

2.2 Extracellular Vesicles

2.2.1 Exosomes: Small Vesicles Participating in Cell Communication and Immune Responses

Communication between most cells mainly involves the secretion of proteins that through receptor binding on neighboring cells modify their behavior. Recent developments in the field of cell communication research shed the light on the role of small membrane vesicles, produced from almost every type of eukaryotic cell, which participate in intercellular communication.[47, 48]
Cells secrete different types of membrane vesicles (Fig. 2.2) including microvesicles, ectosomes, apoptotic bodies and exosomes which vary in size, morphological characteristics and functions.[49, 50]

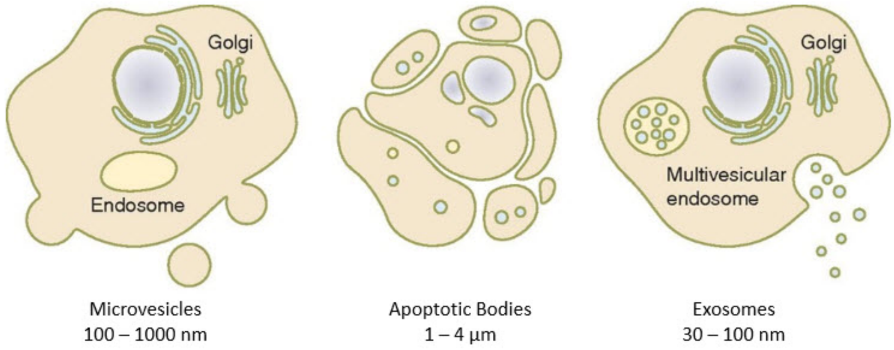

FIGURE 2.2: Different classes of extracellular vesicles. Microvesicles derive from outward blebbing of the cell membrane with subsequent release of the vesicles. Apoptotic bodies is the result of late stage apoptosis. Exosomes are released after fusion of the multivesicular endosomes with the plasma membrane. Modified from [51]

The current project focuses on the exosomes for their peculiar physiological characteristics. Exosomes are small membrane vesicles limited by a lipid bilayer which size span from 30 to 100 nm and contain certain combinations of lipids, adhesion and signaling molecules as well as mRNAs and microRNAs.[13]
As illustrated in Fig. 2.3, their biogenesis involves the formation of intraluminal vesicles by inward budding of the limiting membrane into the lumen of late multivesicular endosomes. As a consequence of the fusion of arising multivesicular bodies with the delimiting plasma membrane, these vesicles are released as exosomes and eventually enter extracellular matrix and body fluids such as blood plasma, urine and saliva.[50, 52]

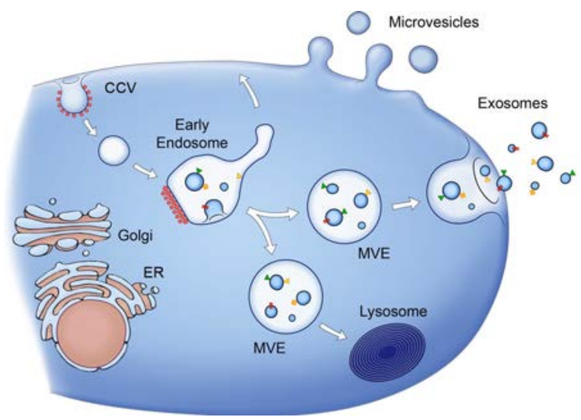

FIGURE 2.3: The biogenesis of extracellular vesicles. Clathrin–coated vesicle (CCV), multivesicular endosome (MVE). Modified from [53]

The best characterized cellular sources of exosomes are immune cells and tumors, and different techniques have been developed in order to detect, isolate and decipher their functions. Depending on their origin, key functions of exosomes include regulation of

immune responses, stem cell maintenance and plasticity, set up of tumor escape mechanisms as well as mediation of regenerative and degenerative processes.[50, 54, 55]
Exosomes are molecular complex organelles participating in intercellular communication with multiple functions which are still under intense investigation. As illustrated in Fig 2.4, possible mechanisms of action may involve functional delivery of microRNAs, anti–inflammatory cytokines and other proteins influencing or modifying the behavior of neighboring cells.[13, 15, 56, 57]

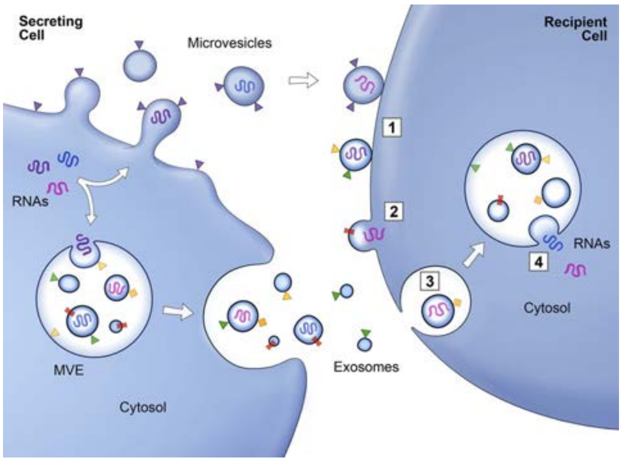

FIGURE 2.4: Protein and RNA transfer by extracellular vesicles. Transmembrane proteins (rectangles), membrane–associated proteins (triangles) and various types of RNAs are incorporated by recipient cells. EVs after being released in the extracellular environment, may either fuse with the recipient plasma membrane (2) or be endocytosed (3). Modified from [53]

2.2.2 ASC–Derived Vesicles as a Novel Approach for Cell–Free Therapy

The therapeutic use of stem cells gave rise to several concerns in the past decade regarding the potential risks for human health. Some of the challenges concerning transplanted stem cells are immune–mediated rejection, loss of function and limited cell survival.[58]
A major problem in utilizing stem cells for clinical applications is the possibility of malignant transformation. The production of a sufficient amount of MSCs for clinical use requires consistent in vitro expansion, which can lead to spontaneous transformation of the cells. In the light of these observations, the possible ways of translating the potential of MSCs to the clinic should be cautiously pondered.[59]
While the prevailing role of MSC paracrine action in tissue repair and immune response modulation has already been established, the role of the extracellular vesicles remains to be studied. The protective paracrine activity of MSCs in kidney injury, intervertebral

disc degeneration and cardiovascular diseases fostered several studies into the potential contribution of MSC–derived microvesicles in the positive therapeutic outcome.[11, 60–63]

In summary, targeting exosomes to hinder their effect in disease, using them for drug delivery or exploiting their natural therapeutic potential are all strategies that appear as promising new tools for the clinical diagnostics and potentially for novel therapeutic approaches.[60, 63–65]

2.3 Standardization in ASC–Derived EV Research

Undoubtedly, extracellular vesicles are central to intercellular communication and potentially, have a great therapeutic capacity. However, despite great effort put into elucidating extracellular vesicle biology, many of the properties and mechanisms attributed remain largely elusive. As a matter of fact, some investigations describe contradictory results, even regarding particles derived and isolated from the same cell types. MSC–derived exosomes, for example, have been shown to both suppress and promote tumor growth and progression.[11, 66]

Such divergences are probably a consequence of differences in cell culture conditions prior EV isolation, differences in the purification protocol adopted or due to lack of consistent extracellular vesicle characterization. In order to clearly delineate the biological roles and therapeutic potential of secreted vesicles, standardized protocols for their purification and phenotypical characterization are urgently needed.[16, 67]

2.3.1 Bovine Serum–Derived EVs

Fetal calf or bovine serum–supplemented growth medium often represents the standard culture environment in the majority of *in vitro* investigations. However, serum of animal or other origin contains high amounts of microparticles and the currently adopted protocols of serum purification fail to completely eliminate all potentially contaminating particles, fact that could undermine both quality and quantity of the isolated ASC–derived exosomes.

In order to avoid contamination of cell–derived particles by serum EVs, two main solutions have been proposed.[68] The first consist in to culturing cells in serum–free growth medium. Such an abrupt switch to nutrient–poor growth medium will unavoidably induce a stress response, which may lead to release of EV of different composition and/or cellular origin. Currently, no side–by–side comparisons have been performed yet in order to assess the effect of the commercially available serum–free growth media to EV quality.[69, 70]

Another solution is to eliminate serum–derived exosomes before cell culture by performing overnight ultracentrifugation at 100,000 x*g*. However, the aforementioned technique results in exosome–free serum, but not in contaminating–particle–free serum, implicating that this fraction is, consequently, harvested along with the conditioned medium under investigation. It is generally not reported whether, non properly vesicle–depleted serum can bias downstream applications regarding EV isolation, analysis and functional translation. Precautions in order to avoid artifactual precipitated vesicles are recommended.[66, 67, 71]

2.3.2 Standardized ASC–Derived Exosome Isolation

The "gold standard" in exosome isolation, commonly adopted in most EV–related investigations, is differential centrifugation followed by ultracentrifugation.[68] However, this technique displays several limitations, in terms of sample purity and particle yield which calls for an alternative, more reliable and easily reproducible solution. Different methodologies have been adopted in order to purify exosomes and microvesicles from MSC/ASC–conditioned culture medium or other biofluids. Size exclusion techniques, immunoaffinity isolation and polymer–based precipitation are some of the alternative methods proposed in order to enrich EVs. Common flaws in the employment of the aforementioned techniques, such as deformation and breakup of large vesicles when passed through filter pores, low particle yield caused by immune selectivity and co–precipitation of contaminating particle fractions may interfere with downstream applications.[16, 67, 72–74] Combination or hybrids of the different isolation methods may result in more efficient recovery of high quality small EVs.[75]

2.3.3 Hypoxia–Conditioned ASCs

The effects of low oxygen concentration on stem cell growth, function and development is well documented.[17, 42, 76, 77] However, the effect of low oxygen concentration on the production and release of exosomes from adipose derived stem cells is still under investigation. In different studies, various mechanisms associated with hypoxia–induced exosome release have been proposed, such as STAT3–mediated signaling and HIF–1α involvement.[71, 78] These classic hypoxia–induced transcription factors seem to play a key role in the activation and enhancement of ASC secretory and particle–sorting pathways, fact that could be exploited in order to increase the yield of secreted particles.

2.3.4 Quantitative and Qualitative Evaluation of EVs

Different optical and non-optical methods have been recently developed or adapted from well–established techniques for the assessment of EV size, concentration and characteristic features of EVs, such as the presence of determined surface markers or proteic and nucleic acid cargo.

Electron microscopy is a conventional optical method proven very useful in EV research. The use of heavy metal stains in transmission electron microscopy (TEM) and the combination of immunoglobulins in immuno–electron microscopy (IEM), provide direct evidence for the presence of vesicular structures with specific EV features.[79]

Another optical method used in EV research is represented by atomic force microscopy (AFM), where the possibility of surface analysis by sub–nanometer resolution is exploited in evaluating EV morphology.[73, 80, 81]

Nanoparticle tracking analysis (NTA), another optical particle tracking method, has developed and continuously improved in recent years and is able to provide both quantitative and qualitative information regarding specific EV populations.[82, 83]

Western blotting is the standard method employed to demonstrate the presence of specific surface proteins, reportedly associated with EVs or EV subpopulations, for instance exosomes. This technique, however, is not suited for EV quantitative determination.[54, 74]

Protein microarrays are powerful tools for proteomic characterization of various sample types. The use of microprints coated with a wide range of capturing antibodies, allow simultaneous detection of a wide variety of different antigens and peptides.[84, 85] A modified and highly sensitive EV array has been successfully employed to capture and characterize exosomes isolated from plasma obtained from healthy donors.[86]

Along with the aforementioned EV assessment methodologies used in secreted–vesicle research, other proteomic and nucleomic techniques, such as mass spectrometry and nuclear magnetic resonance (NMR), arise as new and powerful tools in the quest of comprehending the nature and functional relevance of the extracellular vesicles.

Chapter 3

Aim & Objectives

In the midst of continuously growing interest in extracellular vesicles, technical standardization is critical, in order to mind the gap between the different approaches adopted in EV study. The influence of these diverse techniques on producing comparable results regarding microparticle characterization still remains unclear.

The aim of the present project, is to investigate and optimize critical aspects of the *in vitro* ASC culture conditions as well as compare currently available particle–isolation methods.

The first objective of the current study, is to investigate the efficiency of a combined particle depletion protocol aimed to produce a high quality particle–free fetal calf serum. Speculating on the reliability of the classic particle–isolation method, lead to the second objective of the present investigation, which is to compare sequential ultracentrifugation to a highly acclaimed commercial solution, aimed to improve the quality and increase the quantity of isolated exosomes.

In the third and final objective of the current study, we investigate whether hypoxic conditioning of ASCs at 1% of O_2 concentration has the ability to enhance the release of exosomes compared to normoxia–expanded (20% O_2) adipose–derived stem cells.

Chapter 4

Materials & Methods

4.1 Production of Exosome–Free FCS

Fetal calf serum (FCS) (Helena Bioscience) was depleted of endogenous exosomes by a three–step procedure. In the pre–clearing phase, the serum was centrifuged at 480 xg for 10 minutes, 2,000 xg for 30 minutes and 10,000 xg for one hour in order to eliminate cell debris, large apoptotic bodies, large vesicles and microvesicles. In each of the above steps the supernatant was carefully aspirated and retained, whereas, the pellet was discarded. The supernatant was afterwards filtered through a 0.22 μm syringe filter (Sarstedt, Numbrecht, Germany) and diluted (10% at most) in RPMI 1640 (GIBCO, Invitrogen). The last step consisted in ultracentrifugation of the clarified and diluted FCS at 100,000 xg for the duration of minimum 18 hours in a pre–cooled (4 ^oC) ultracentrifuge (fixed–angle rotor RP70T, Beckman Coulter, Brea, CA, USA).

4.2 ASC Cultures

Human ASCs isolated from 4 different donors (designated respectively 12, 21, 23 & 24; isolated from lipoaspirates by the Laboratory for Stem Cell Research, Aalborg University) were used to isolate exosomes. They were maintained and expanded in αMEM (GIBCO) supplemented with 10% fetal calf serum (FCS) (Helena Bioscience), 10 KUnits/mL penicillin, 10 mg/mL streptomycin and 5 mg/mL gentamicin (all supplemented products were from Sigma Aldrich, St Louis, MO, USA) until they reached confluence between 30 and 40% (the ideal relation between exosome release and cell density was determined by titration as described later on). Subsequently, the cell cultures were gently washed with phosphate–buffer saline (PBS) (Lonza) and media substituted by RPMI 1640 (GIBCO) supplemented with 10% exosome–free FCS, for an additional period of

24 hours prior exosome isolation. Duplicates of each cell batch were allowed to grow and expand both under normoxia (20% O_2, 5% CO_2 and 37 ^oC) and hypoxia (1% O_2). Hypoxic expansion of ASCs was performed within an XVIVO System (Biospherix) at 1% O_2 and 37 ^oC in a 5% CO_2 humidified environment. ASCs were seeded with a cell density of 5,000 cells/cm^2 and the medium was replaced with fresh, pre–warmed growth medium every three days until reaching the desired cell confluence.

4.2.1 Relation Between Cell Confluence & Exosome Release

Prior exosome isolation, a titration assay was performed in order to define the optimal relation between cell confluence and exosome release. ASCs 21, 23 & 24 were maintained in αMEM growth medium supplemented with 10% fetal calf serum. Duplicates of each cell batch were seeded at a density of 5000 cells/cm^2 in 6–well cell culture plates and kept at 37 ^oC under both normoxic (20% O_2) and hypoxic conditions (1% O_2). Cell culture media was then substituted by RPMI 1640 supplemented with 10% exosome–free FCS, 24 hours before exosome isolation and cell count. Three different time points, approximately 2, 4 and 6 days after initial cell seeding were chosen and conditioned media was harvested. These different time points represent, respectively, an approximate cell confluence of 30, 60 and 80% respectively. Exosomal fractions from the different samples were assessed by Nanoparticle Tracking Analysis (NTA) and the number of cells was determined with the use of PicoGreen dsDNA quantitation assay (Invitrogen, Molecular Probes, OR, USA). Exosome release was normalized by cell and the relation between cell confluence and exosome release/cell was then plotted against both the percentage of cell confluence and cell density.

4.3 Exosome Isolation

To assess exosome release, ASCs were cultured as described above long enough to allow cells to attach and achieve a growth phase. After culture in normoxia or hypoxia in RPMI 1640 growth medium, supplemented with 10% exosome–free FCS, for the duration of 24 hours, conditioned media (CM) was harvested for exosome isolation. Prior exosome isolation, CM was pre–cleared by centrifugation (480 xg for 10 minutes, 2,000 xg for 20 minutes and 10,000 xg for 30 minutes) in order to pellet apoptotic bodies, microvesicles and cell debris. Supernatant was then carefully collected and filtered through a 0.22 μm syringe filter. Successively, CM was concentrated using centrifugal filter devices with a nominal molecular weight limit (NMWL) of 100,000 (Amicon Ultra-15 Centrifugal Filter Devices, Millipore) in order to maximize the yield of the isolated exosomes. Total protein concentration of the samples, before and after the particle isolation procedures, was assessed by BCA protein assay (described later on). Figure 4.1 B depicts the two different methods used in order to fractionate the ASC–derived secretome.

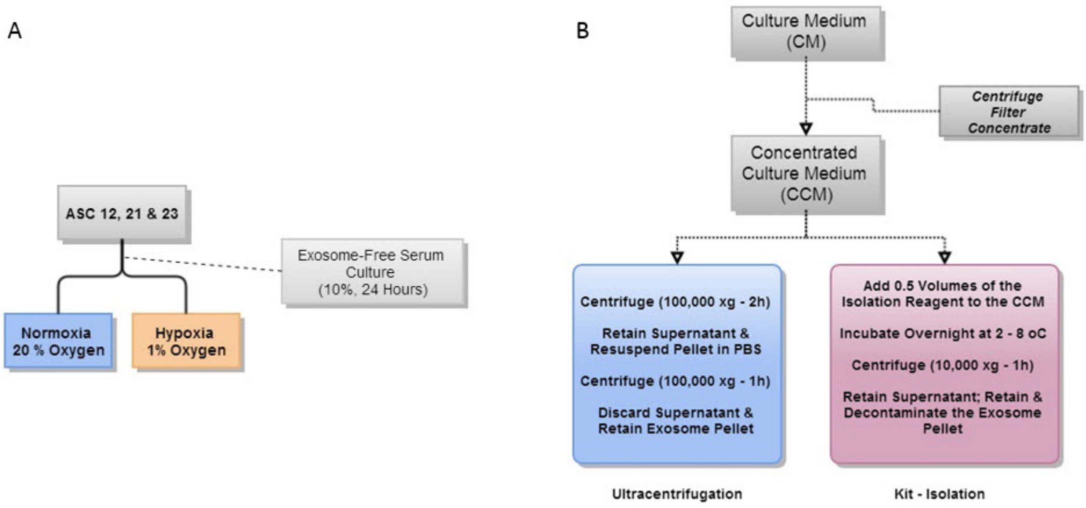

Figure 4.1: ASC lines 12, 21 and 23 upon reaching 30–40% confluence, were gently washed and conditioned for 24 hours in exosome–free growth medium prior harvesting culture medium (A). ASC–derived secretome fractionation and exosome isolation workflow (B).

All cell cultures employed to isolate EVs, were thoroughly controlled in order to assure that no contamination had occurred (qualitative assessment by optical and electron microscopy) and cell viability exceeded 95% (as determined by Trypan blue exclusion).

4.3.1 Polymeric Precipitation

Exosome precipitation with the Total Exosome Isolation reagent (Invitrogen) was performed according to the manufacturer's instructions. Briefly, the concentrated culture medium (CCM) was incubated with the isolation reagent overnight at 2–8 ^{o}C and centrifuged at 10,000 xg for one hour. Removal of unincorporated dye from labeled exosome preparations was achieved with the use of spin columns (Exosome Spin Columns, Invitrogen). Pelleted exosomes were resuspended either in PBS or RPMI 1640 containing 10% exosome–free FCS and stored at -20 ^{o}C.

4.3.2 Sequential Ultracentrifugation

The pre–cleared, concentrated sample was centrifuged in a pre–cooled (4 ^{o}C) ultracentrifuge (fixed-angle rotor RP70T, Beckman Coulter) at 100,000 xg for two hours to pellet the exosomes. The supernatant was retained and the collected exosomes were washed once in PBS by ultracentrifugation at 100,000 xg for one hour.

4.4 Protein Quantification (BCA Protein Assay)

Protein contents were measured using a BCA protein Assay kit (Pierce BCA Protein Assay Kit, Thermo Scientific). BCA Protein Assay is a detergent–compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total protein. An aliquot of every sample, re–suspended in PBS, was mixed with RIPA buffer (Sigma Aldrich) supplemented with a cocktail of protease inhibitors (Roche Diagnostics, Mannheim, Germany). The diluted sample, was then sonicated for 5 minutes, three times, with vortexing in between, in order to disrupt the membranes and enhance the release of protein content from the extracellular vesicles. After incubation for 30 minutes at 37 ^oC with the working reagent, the samples were analyzed with the NanoDrop (ND–1000 Spectrophotometer, Fischer Scientific, Hampton, USA) at 562 nm and the results elaborated by the ND–1000 v3.1.0 software (Fischer Scientific). Protein concentrations were determined and reported with reference to standards of a common protein, in this case represented by bovine serum albumin.

4.5 Exosome Characterization

4.5.1 Nanoparticle Tracking Analysis (NTA)

Nanosight LM10–HS (Amesbury, UK) was employed in order to analyze, in both qualitative and quantitative fashion, the exosomal fraction isolated from the conditioned supernatants. Briefly and according the manufacturer's instructions, the instrument was blanked with 50 nm silica microspheres, followed by analysis of both exosomal and soluble fractions of cell culture origin, obtained under both normoxic and hypoxic conditions. The samples were diluted (dilution factor 200) in DPBS without Ca^{2+} or Mg^{2+} (Lonza), previously filtered through a 0.22 μm syringe filter. The camera gain and camera shutter speed were set, respectively at 350 and 700. The duration of the particle movement capture was set to 60 seconds.
During NTA measurement, particles (in this case, exosomes) are illuminated by a focused laser beam passed through particles in suspension. The light scattered by each individual particle in the field of view is focused by the microscope onto the image sensor of the video camera. The NTA software (version 2.3, build 013) identifies and tracks each particle, thus enabling measurement of the mean square displacement (MSD) of

particle movement, which is used together with the temperature and the viscosity of the liquid containing the particles to calculate particle size through the Stokes–Einstein equation.
Size distribution profiles obtained from NTA were averaged within each sample across the video replicates, and then averaged across samples to provide representative size distribution profiles. These distribution profiles were then normalized to total nanoparticle concentrations.

4.5.2 Atomic Force Microscopy (AFM)

For AFM imaging of the exosomes, a solution at a concentration of approximately 0.8 mg/mL was placed on a freshly cleaved mica surface (EMS Inc.). In order to attract the particles on the mica surface the substrates were treated with 3-Aminopropyl Trimethoxysilane-Tetramethoxy-Silane (APTMS) (Sigma Aldrich) vapor to create a positively charged surface. Briefly, the plates were heated up to 100 ^{o}C in a desiccator containing a test tube filled with 0.7 ml toluene and 0.1 ml APTMS. The desiccator was evacuated down to 50 mbar, filled with argon and left for 45 minutes. The APTMS–treated mica plates were used immediately after modification.
After 5 minutes of incubation the mica disc was blown dried under a stream of nitrogen and placed in the microscope. All measurements were performed on a Veeco Multimode IIIa Atomic Force Microscope (Veeco Metrology, Santa Barbara, CA) in tapping mode using OMCL–AC160TS cantilevers (Olympus, Japan). Data were analyzed using free image processing software WSxM (Nanotec Electronica S.L., Madrid, Spain).

4.5.3 Transmission Electron Microscopy (TEM) with Immuno–Labeling (IEM)

Initially, negative staining was performed by applying 5 μL of each sample to the surface of a carbon coated, glow discharged 400 mesh Ni grid. After two minutes the grid was stained with 3 drops of 1% phosphotungstic acid (PTA), pH 7.9 and blotted dry on filter paper.
A 5 μL aliquot of each exosomal suspension was placed on a carbon–coated, glow–discharged 400 mesh Ni grid for two minutes. Grids were washed in two drops of PBS and blocked for 5 minutes at room temperature in one drop of PBS containing 0.5% ovalbumin. Three mAbs (anti-CD9, CD81 from LifeSpan Biosciences, Inc., WA, USA and anti-CD 63 from BioLegend, CA, USA; diluted 1:5) were used as primary antibodies. The carbon coated grids were allowed to incubate with the primary antibodies for one hour at 37 ^{o}C. Following incubation, the grids were washed in 3 drops of PBS

and incubated with anti–mouse serum conjugated with colloidal gold particles of 15 nm diameter (British BioCell, UK) which was used as the secondary antibody. The gold particles were diluted 1:20 with 1% cold fish gelatin for one hour at 37 ^oC. The grids were then washed in 3 drops of PBS followed by wash in 2 drops of 1% cold fish gelatin for 10 min each. The grids were finally washed in 3 drops of PBS, stained with 2 drops of 0.5% PTA and blotted dry.

Electron microscopy was performed on a JEOL 1010 electron microscope operated at 60 kW. Images were taken using an Olympus KeenView digital camera. For size determination, a grid–size replica (2160 lines/mm) was used.

4.5.4 Extracellular Vesicle Array

The Extracellular Vesicle (EV) Array is a highly sensitive tool capable of detecting and phenotypically characterizing a variety of extracellular vesicles in samples with cell culture–derived exosomes among others. In brief, a microarray print (SCHOTT Nexterion, DE, USA) with duplicate spots of 16 different biotinylated antibodies (16 antibody spots; two positive controls; one negative control) was used to capture and characterize exosomes from the cell culture supernatants. Among others, a cocktail of antibodies against the tetraspanins CD9, CD81 (both from LifeSpan Biosciences) and CD63 (BioLegend) was selected in order to ensure that all exosomes captured are detected in a concomitant exclusion of other types of vesicles.
Samples were derived from three different batches of ASCs (12, 21 & 23), cultured and expanded under both normoxic (20% O_2) and hypoxic conditions (1% O_2). The exosomal fractions were isolated with the use of exosome isolation kit and their characterization with EV Array was opposed to that of concentrated and untreated samples from the same batches, used as controls. Figure 3.2 displays, in a graphical manner, the principle of EV Array.

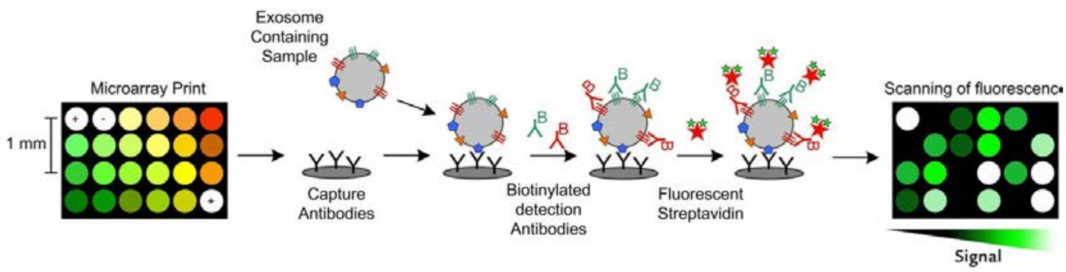

FIGURE 4.2: Extracellular vesicle detection with the use of a customized EV Array. The captured exosomes are detected with a cocktail of biotinylated antibodies against tetraspanins CD9, CD63 and CD81 followed by fluorescent streptavidin staining. Modified from [86]

4.6 Statistical Analysis

Statistical significance was evaluated, with the help of GraphPad Prism 5 (GraphPad Software), using one–way Anova with a Tukey's multiple comparison test and a confidence interval of 95% for all samples. To estimate correlation, a Spearman–ranked correlation test was performed. Student t test was used to compare two groups. All data is presented as mean ± SEM. Asterisks represent statistically significant difference ($P<0.05$).

Chapter 5

Results

5.1 Background

One of the major challenges in the field of extracellular vesicle (EV) research is to improve and standardize methods for EV isolation and analysis. EV isolation can be achieved by a variety of methods such as ultracentrifugation and polymeric precipitation.
Prior particle isolation and their characterization it was necessary to define the optimal cell culture conditions in order to maximize the yield and purity of the isolated particles. For this purpose, it was investigated the effect of oxygen concentration and cell density on the exosome production and release by ASCs.

5.2 Production of Exosome–Free FCS

In order to avoid contaminating particles of animal origin, the serum was pre–treated, as previously described, in order to eliminate the vast majority of interfering particles. Three independent assays confirmed the validity of the protocol adopted in order to eliminate FCS–derived extracellular vesicles.
Fig 5.1 illustrates the three distinct phases resulting after 18 hours of ultracentrifugation of the diluted fetal calf serum. The upper part of the polycarbonate ultracentrifuge tube contains the exosome–free FCS and this is the fraction of clarified serum used in order to grow and expand the ASCs. Suspension phase represents about 10% of the total tube volume and has a thicker consistency. Aliquots of the pellet and suspension phase were analyzed by NTA and consequently discarded as they contained the bulk of contaminating particles.

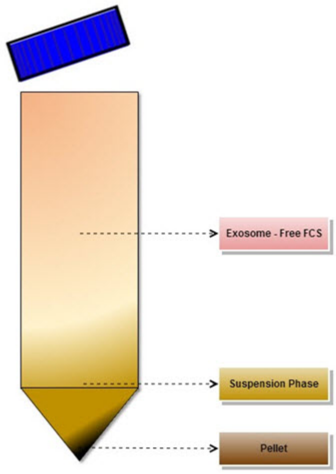

FIGURE 5.1: Schematic representation of the different phases resulting after ultracentrifugation at 100,000 xg of the clarified and diluted FCS.

Following characterization, by NTA, of the different components of the ultracentrifuged serum, it was able to identify and describe in both qualitative and quantitative fashion the different particle populations contained in the FCS.

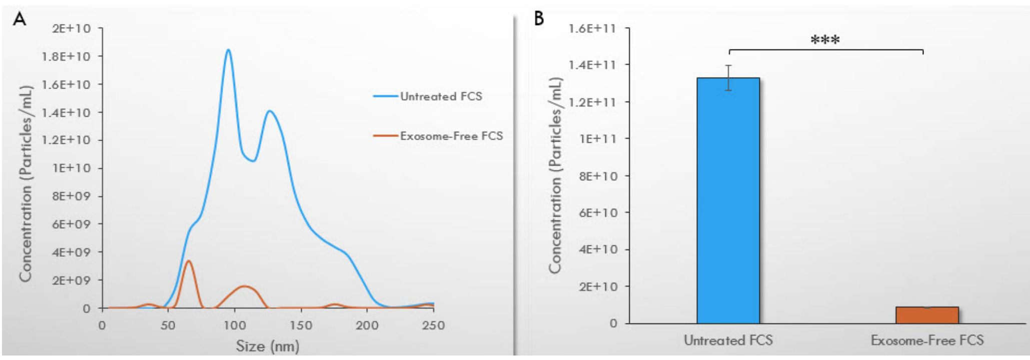

FIGURE 5.2: Comparative illustration of the size distribution (A) and the area under the curve (B) of the untreated and exosome–free FCS–derived EV fractions, analyzed by NTA.

As displayed in Fig 5.2, by comparing untreated FCS to the purified from vesicles sample, it can be observed that both the concentration (Fig 5.2 B) and the size of the particles (Fig. 5.2 A) remaining in the treated sample are significantly minor. The size of the vesicles contained in the untreated serum span from roughly 50 nm to over 200 nm in diameter. On the other hand, exosome–free FCS contains markedly less particles and their mean size does not exceed 86 nm of diameter.

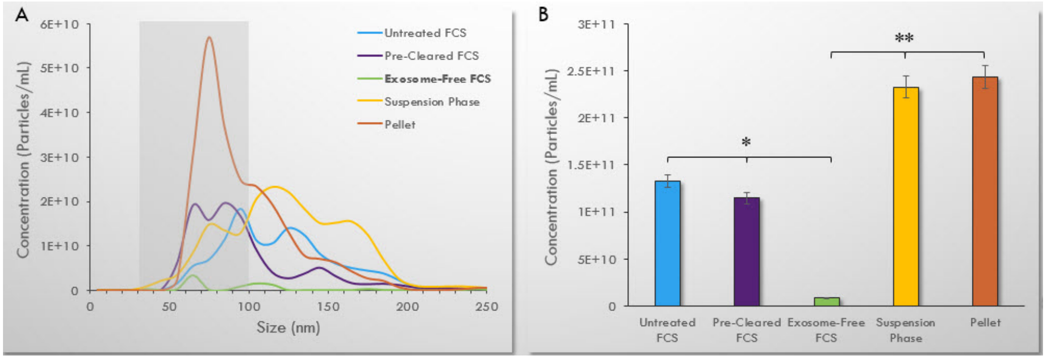

FIGURE 5.3: Comparative illustration of the size distribution (A) and the area under the curve (B) of the EV fractions contained in FCS and separated by differential centrifugation and ultracentrifugation, as assessed by NTA. The highlighted area on the left graph (A) represents the distribution area of the exosomes, which size span from approximately 30 to 100 nm of diameter.

Fig. 5.3 displays, in detail, the size distribution and the relative concentration of the different fractions resulted from the FCS purification process. As it can be observed on Fig. 5.3 A, after the first step of differential centrifugation, the pre–cleared serum displays a narrower particle size distribution, as compared to the untreated sample and a mean size of 95 nm in diameter. Following filtration and ultracentrifugation, the suspension phase and the pellet result in almost two distinctive particle populations (Fig 5.3 A). The resulting pellet displays characteristics similar to those of the exosomes such as a size distribution that span from roughly 50 nm to 100 nm of diameter and a mean size of 95 nm. Suspension phase resembles EVs similar to microvesicles and their size spread from 30 to 200 nm with a mean size of 137 nm.
After carefully aspirating the supernatant above the suspension phase and discarding the remaining phases we manage to obtain a highly purified serum, virtually free from any contaminating particles. Although there are few particles left in the purified serum, exosome–free FCS contains significantly less particles compared to both untreated serum and discarded fractions resulting after ultracentrifugation (Fig 5.2 B and 5.3 B).

5.3 Exosome Isolation

5.3.1 Polymeric Isolation vs Ultracentrifugation

The classic approach of exosome isolation by differential centrifugation and ultracentrifugation compared to that of a commercially available exosome–isolation kit, resulted in exosomal fractions of diverse quality and quantity.
The initial amount of conditioned media used in order to fractionate the stem cell secretome was 60 mL per cell batch and identical amounts were employed in both isolation

techniques. Cell viability exceeded 95% and no contamination was observed in the harvested culture supernatants.

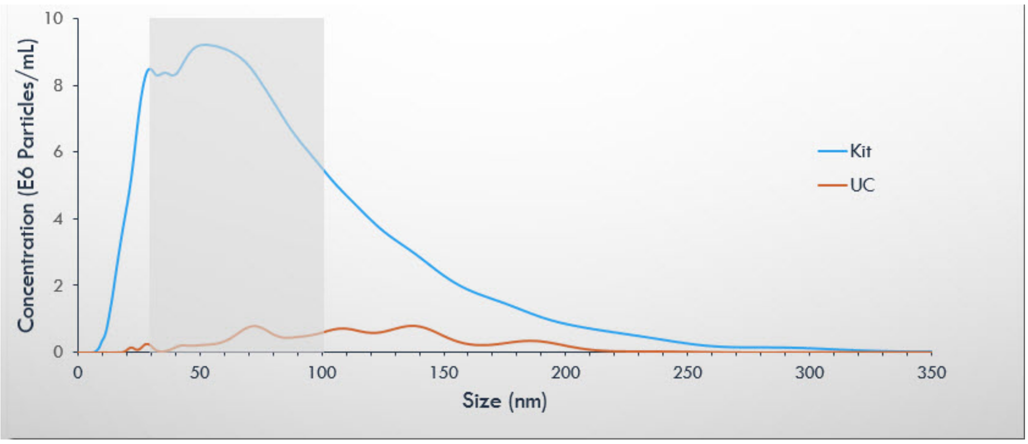

FIGURE 5.4: Size distribution profiles of the two isolation methods adopted: polymer–based exosome isolation (Kit) and ultracentrifugation (UC). The highlighted area on the graph represents the distribution area of the exosomes, which size span from approximately 30 to 100 nm of diameter. All values displayed, are corrected to the background given by exosome–free growth medium (EFM) and RPMI 1640, used to grow the ASCs.

Fig. 5.4 illustrates the size distribution profiles of the exosomal populations isolated either by kit or ultracentrifugation. Significant differences can be observed between the two isolation methods, both regarding the size distribution and the amount of the isolated particles. As delineated by the size distribution profiles, the kit produced a more uniform population of particles compared to the particles isolated by ultracentrifugation. Kit–isolated particles correspond to that of the exosomes which size span from roughly 30 to 100 nm of diameter with an average mode of approximately 77 nm (Fig. 5.5 B). On the other hand, ultracentrifuged particles had a more ample size distribution (between 20–200 nm) with an average mode of 116 (Fig. 5.5 B) and a significantly lower concentration (Fig. 5.5 A).

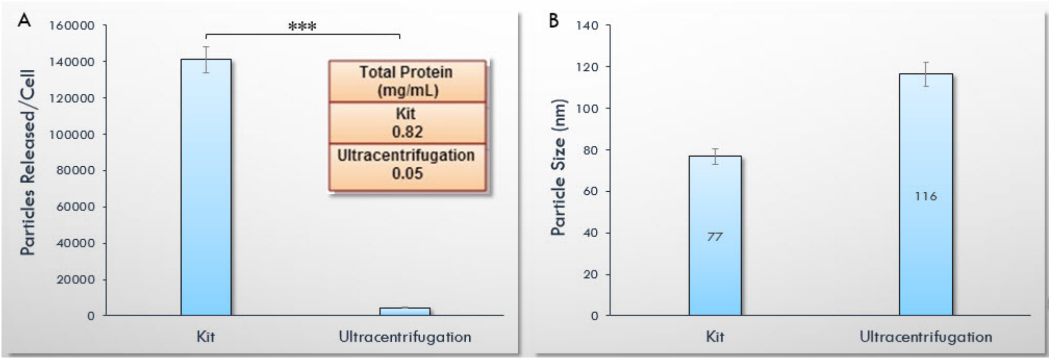

FIGURE 5.5: Comparative illustration of the two isolation methods adopted: polymer–based exosome isolation (Kit) and ultracentrifugation. All values displayed, are corrected to the background given by exosome–free growth medium (EFM) and RPMI 1640 used to expand the ASCs. The values in the center of the columns (B) display the average diameter (in nm) of the isolated particles among the cell batches under investigation.

Fig 5.5 A displays, in comparative fashion, that the concentration of particles isolated by the kit is significantly higher than by sequential ultracentrifugation.

Downstream applications, such as western blotting, protein microarray techniques and particle visualization by transmission electron microscopy or atomic force microscopy require knowledge of the total protein counts from the samples under examination, in order to comply to the minimum limits of detection of the various techniques.

Total protein counts, as determined by BCA protein assay, for the isolated exosomal fractions are shown in the panel on Fig. 5.5 A. Total protein counts were normalized by subtracting the protein counts given by the growth medium. BCA protein assay measurements revealed a mean difference in the exosome yield (in terms of total protein counts) of about 93% in favor of the precipitation technique.

5.4 Oxygen Concentration & Exosome Release

In order to investigate and assess the effect of low–oxygen–concentration on exosome release form ASCs, four different batches (12, 21, 23 and 24) were allowed to grow and expand under both normoxic (20% O_2) and hypoxic (1% O_2) conditions in a 5% CO_2 humidified environment.

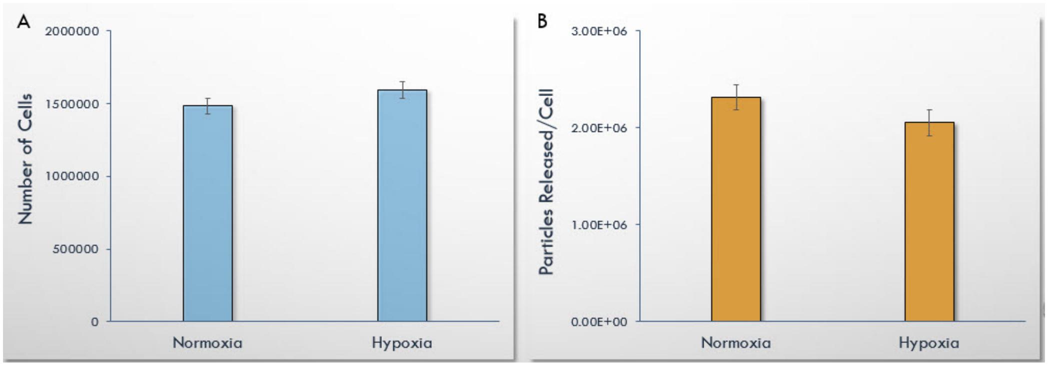

Figure 5.6: Comparative illustration of cell proliferation (A) and exosome release (B) by ASCs 12, 21, 23 and 24 under both normoxic and hypoxic conditions.

Figure 5.6 illustrates the effects of oxygen concentration on cell proliferation and release of exosomes by ASCs. After 24 hours, the number of cells expanding under hypoxic conditions is slightly higher than the cells growing in normoxia (Fig. 5.6 A). In the same arc of time, the particles released in the supernatant is to some extent higher under normoxic conditions than in hypoxia (Fig. 5.6 B). All values, regarding particle release, are normalized per single cell EV–release.

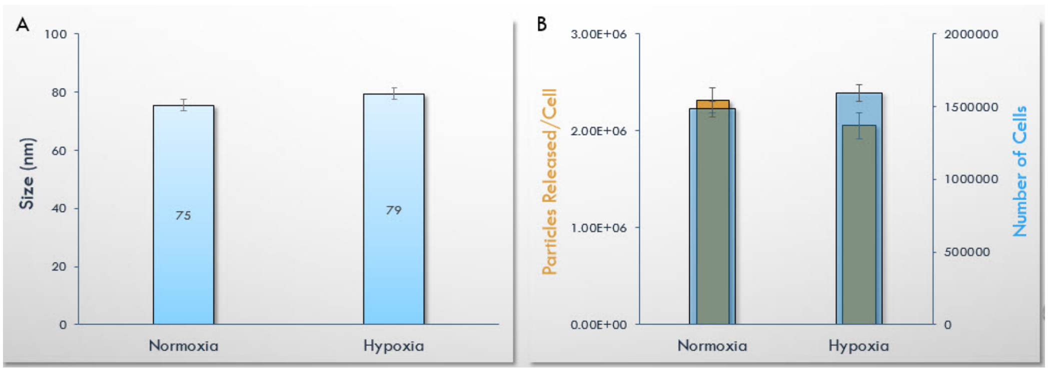

Figure 5.7: Comparative illustration of the mean particle size (A) and the relation between EV release and number of cells (B) expanded under both normoxic and hypoxic conditions.

The mean size of the isolated particles, as displayed by Fig 5.7 A, corresponds to that of the exosomes, i.e. 75 and 79 nm for particles released respectively in normoxia and hypoxia. When relating cell numbers and respective EV release, as illustrated in Fig. 5.7 B, it can be observed that exosome release and correspondent amount of cells in the cell culture flasks are inversely proportional. As expected, after 48 hours of culturing, the amount of cells in the culture flasks was higher than in the 24–hour condition. However, the release of exosomes after 48 hours conditioning (as determined by NTA) was lower in all cell cultures under investigation.

5.5 Cell Confluence & Exosome Release

In order to determine the relation between growth area covered by the expanding cells and exosome release, a titration assay was performed as previously described. Briefly, ASCs 12, 21, 23 and 24 were cultured, in duplicate, both under normoxic and hypoxic conditions in 6–well culture plates. Supernatants were harvested and analyzed in three distinct time points.

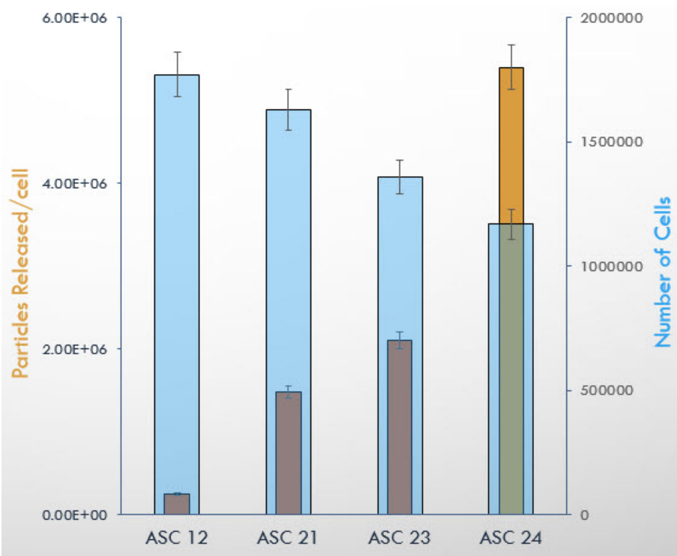

FIGURE 5.8: Comparative illustration of the exosome release by ASC lines 12, 21, 23 and 24 related to the amount of cells present in the respective cell culture flasks after 24 hours preconditioning in exosome–free FCS.

Fig. 5.8 illustrates the correlation between the number of exosomes released by a single cell and the appertaining amount of cells present in the culture flasks after harvesting the conditioned medium. A clear trend can be observed in all cell lines, where higher number of cells present in the culture flask is related to lower amount of exosomes released and isolated by polymeric precipitation. The same trend is observed both under

normoxic and hypoxic conditions.
After relating the area covered by the expanding cells (expressed as cell density) and the particles released, as displayed in Fig. 5.9, it becomes clear the influence of cell density on the number of exosomes released.
The relation between particle release and cell confluence was assessed both in normoxia (20% O_2) and hypoxia (1% CO_2). For low cell densities the number of exosomes released per single cell is markedly bigger than in higher densities, effect that somehow is normalized for higher cell densities. However, in both cases, under normoxic conditions the number of particles released by a single cell is markedly higher than under hypoxic conditions.

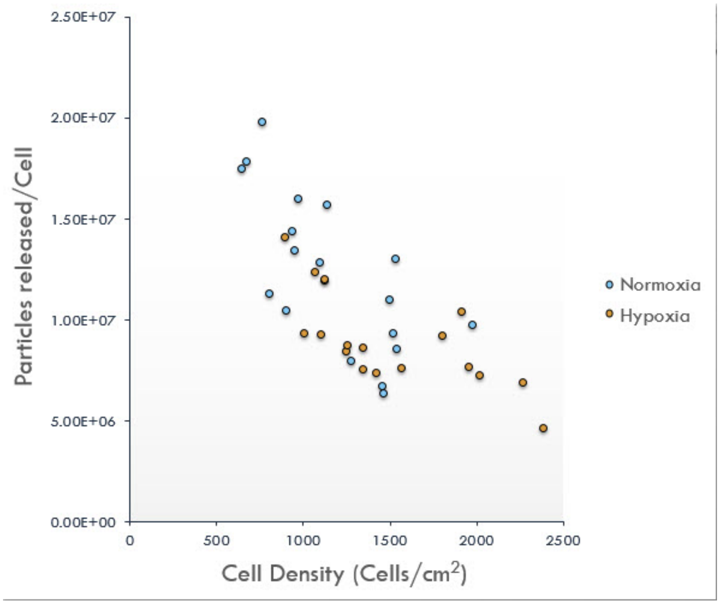

FIGURE 5.9: Comparative illustration of the exosome release related to cell density of ASCs 21, 23 and 24, expanded both under normoxic and hypoxic conditions. All cell lines were pre–conditioned for 24 hours in exosome–free FCS.

As observed from the behavior of all cell batches under investigation, prolonged culturing times had a negative impact in the release of EVs, resulting in a lower yield of isolated particles.

5.6 Quantitative & Qualitative Assessment of Exosomes

5.6.1 Nanoparticle Particle Analysis

NTA, as previously described, is a highly sensitive technique capable of both quantitative and qualitative assessment of the particles contained in a given sample.
Fig 5.10 displays the size distribution profiles (A) and the respective calculated concentrations (B; area under the curve) of the different exosomal fractions isolated from ASC 12, 21, 23 and 24 (expanded under normoxic conditions). All values displayed are corrected to the background given from the exosome–free growth medium used to expand the cells.

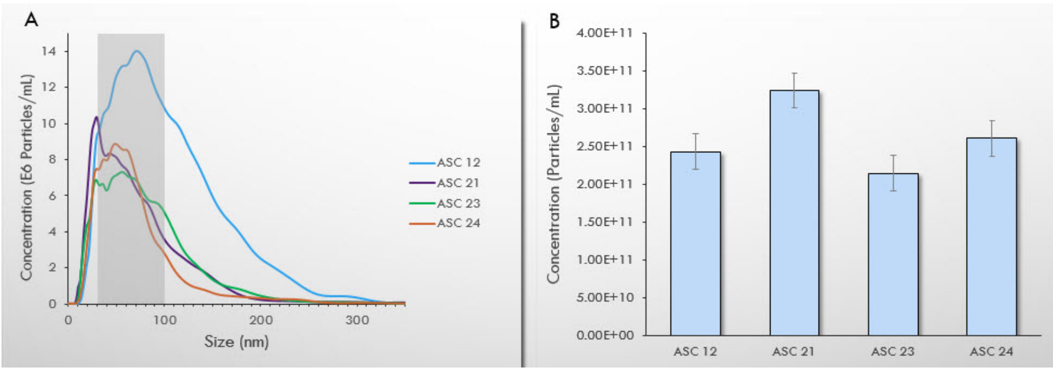

FIGURE 5.10: Comparative illustration of the size distribution and the area under the curve of the ASC 12, 21, 23 & 24 exosomal fractions, isolated by polymeric precipitation. The highlighted area on the left graph (A) represents the distribution area of the exosomes, which size span from approximately 30 to 100 nm of diameter.

Under all experimental conditions, similar size distribution is observed among the different cell lines (A). The relatively congruent amount of particles isolated from the ASCs under investigation (B) confirms the effectiveness of the polymeric EV–isolation.

5.6.2 Atomic Force Microscopy

The ability of AFM to investigate properties of surfaces with sub–nanometer resolution can be exploited to assess EV morphology. Fig. 5.11 illustrates three high resolution AFM images of the exosomal fraction isolated from ASCs 23, cultured and expanded under hypoxic conditions. Although exosomes slightly deformed on the substrate, the images correlate well with the exosome structures obtained from electron microscopy imaging. In the images displayed in Fig. 5.11 we can be observe round, spherical shaped exosomes which size span from 30 to 100 nm of diameter. No differences were observed

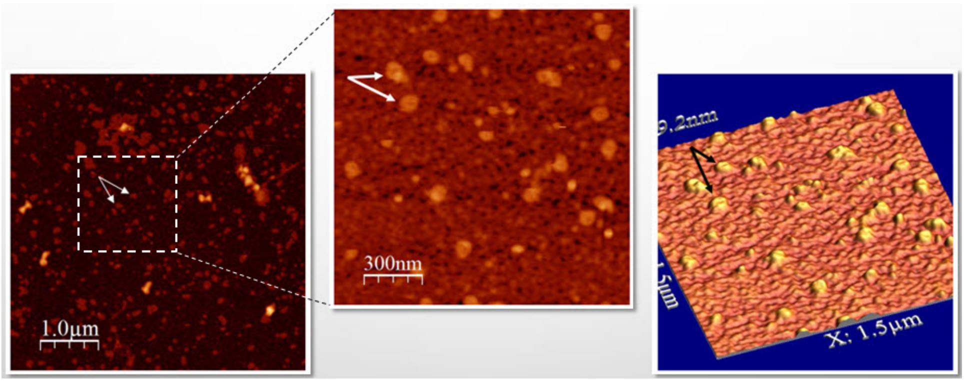

Figure 5.11: AFM image of exosomes (indicated by white and black arrows) on mica substrate, with 3D topography inserted.

between the different cell lines, conditioned both under normoxia and hypoxia (refer to Appendix A for visual comparison of particles isolated under different experimental conditions).

5.6.3 Electron Microscopy

Electron microscopy (EM) techniques are well established and their use in extracellular vesicle research provide direct evidence for the presence of any vesicular structures.

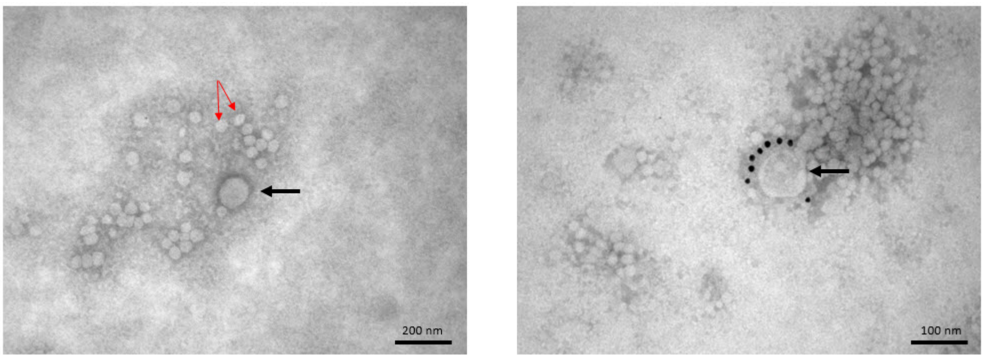

Figure 5.12: Transmission electron microscopic pictures of exosomes (indicated by black arrows). Normal TEM picture on the left (red arrows indicate contaminating lipoprotein particles) and TEM combined with immuno–labeling on the right (IgG–coupled gold nanoparticles are displayed as black round spots).

Fig. 5.12 illustrates the detected, by TEM and IEM, exosomes contained in conditioned supernatant harvested from ASC 23 cultures, expanded under normoxic conditions (exosome isolation by kit). No differences were observed among isolated exosomal fractions from the three cell lines under examination, notwithstanding the culture conditions (normoxia, hypoxia). The two different exosome isolation techniques reveal considerable differences in sample purity. Polymer–based isolated exosomal fractions contained higher amounts of lipoproteins (as indicated by the red arrows in Fig. 5.12) and protein aggregates, as compared to the samples treated solely by ultracentrifugation.

5.6.4 Extracellular Vesicle Array

Techniques such as NTA, AFM and TEM are limited to define the microparticle size range and to estimate microvesicle concentration. Using microarray printed with cell–type–specific antibodies, it is possible to identify specific subsets of extracellular vesicles as, for instance, exosomes.

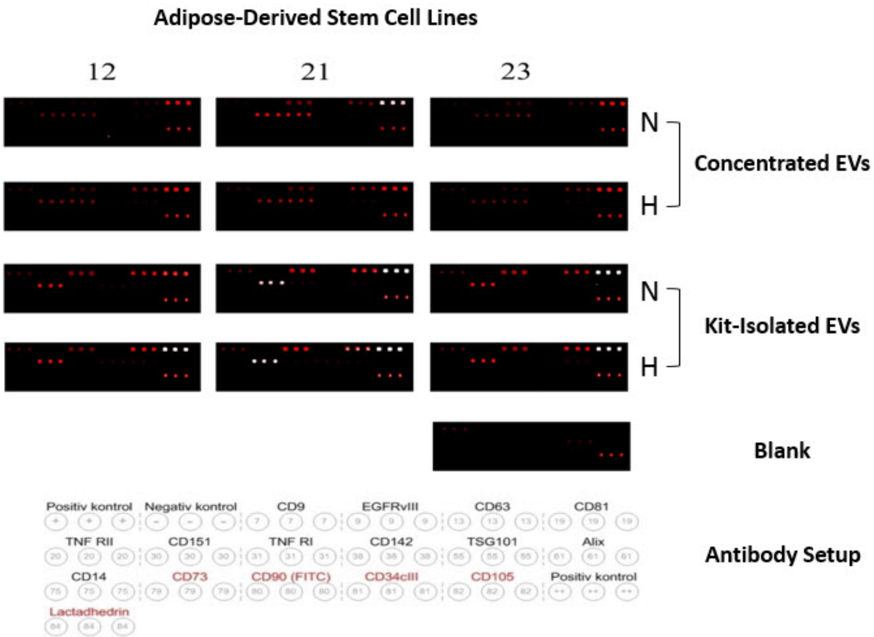

Figure 5.13: EV Array plate setup for the three adipose–derived stem cell lines. Kit–isolated extracellular vesicles (EVs) were compared to concentrated supernatants from the same cell batches. ASC 12, 21 and 23 were cultured under both normoxic (N) and hypoxic (H) conditions. The exosomes were profiled with the use of an EV Array printed with 16 different capturing antibodies.

As displayed in Fig. 5.13, kit–isolated exosomes and untreated concentrated extracellular vesicles (control samples), derived from both normoxic and hypoxic conditioned culture medium, were applied to a panel of 16 different cellular surface antigens. Expression of the specific surface markers was measured as relative fluorescence intensity and plotted against the different markers under investigation.

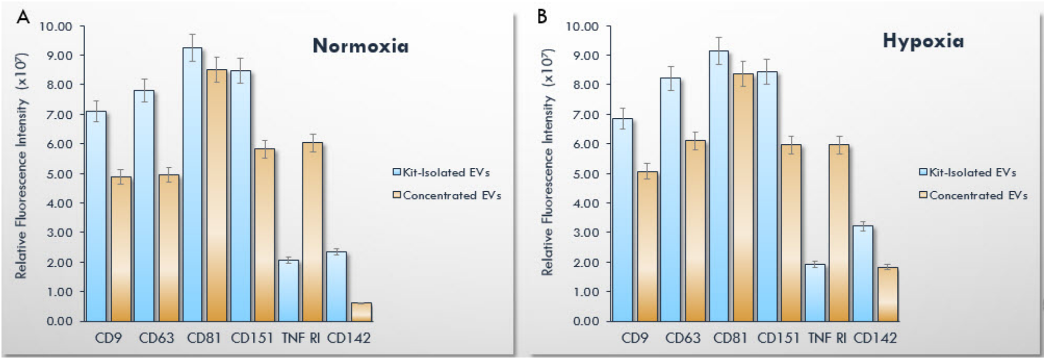

FIGURE 5.14: EV analysis of kit-isolated microparticle fractions as compared to untreated samples obtained both from normoxic (A) and hypoxic (B) conditioned medium. The relative fluorescence intensities are plotted against the surface markers, under investigation, from the different experimental conditions.

Fig. 5.14 shows heterogeneity in the expression levels of individual markers among the different experimental conditions. Under both normoxic and hypoxic growth conditions, the expression of all investigated markers is markedly higher in the kit–isolated samples compared to the untreated, concentrated samples.
The protein profiles of the exosomes bring to light the fact that the tetraspanins CD9, CD63, CD81 and CD151 are expressed at approximately equal levels in the three cell batches under investigation. Tissue factor (CD142) is, generally, expressed in low levels among the tested samples. Concentrated, untreated samples, for instance, display a lower expression compared to that from the kit–isolated particles. TNF RI is highly expressed in the up–concentrated samples compared to the very low expression levels seen in the kit–isolated particles.

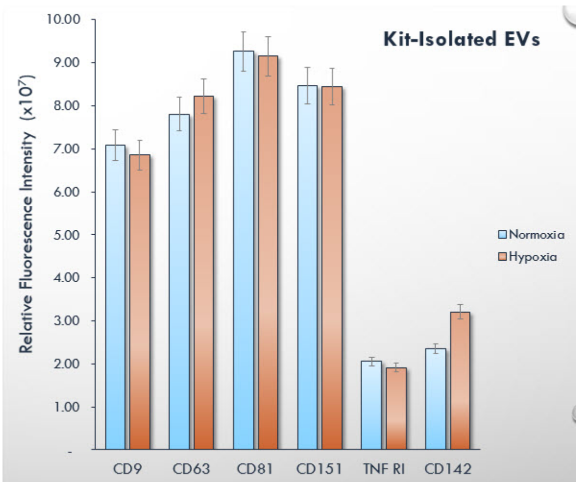

FIGURE 5.15: EV analysis of microparticle fractions isolated by polymeric precipitation. The relative fluorescence intensities are plotted against the surface markers, under investigation, from the different experimental conditions.

As illustrated in Fig. 5.15, no substantial differences are observed between polymeric–isolated samples derived either from normoxic or hypoxic conditioned media.
Fig. 5.10 A displays that the total level of microparticles in the cell culture supernatants noticeably differ, therefore prior to cluster analysis a log2 transformation was required to perform.

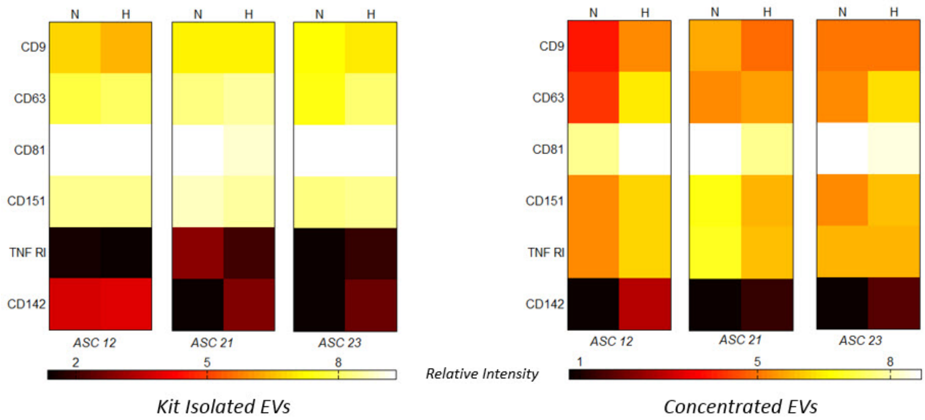

FIGURE 5.16: Phenotyping summary of the exosomal (positive for CD9, CD63 and CD81) population (here referred as extracellular vesicles; EVs) in supernatants from three cell lines. Polymeric isolated exosomes are compared to that of concentrated supernatants, harvested both under normoxic (N) and hypoxic (H) conditions. The relative fluorescence intensity was log2 transformed and a hierarchical clustering was performed for that purpose.

For each cell batch and experimental condition, no considerable heterogeneity is observed in the expression level of the individual surface markers (Fig. 5.16). Expression of the exosome–specific markers CD9, CD63 and CD81 as well as of the tetraspanin CD151 is, in average, 2 fold higher in the kit–isolated samples compared to the untreated samples. TNF RI and CD142 (tissue factor) are, in average, expressed in lower levels in the polymer–based isolated exosomes than in the concentrated EVs.

Chapter 6

Discussion & Conclusions

6.1 Calf Serum–Derived Extracellular Vesicles

In the present investigation, a combined protocol consisting in differential centrifugation, followed by filtration and ultracentrifugation, was adopted in order to purify FCS. The resulting serum contained significantly less particles when compared to untreated serum, thus enabling us to avoid the bulk of contaminating particles that may interfere with both quantitative and qualitative EV analysis. The current particle depletion protocol provides a relative particle–free growth environment to the cells under examination, without depriving them from the necessary trophic factors contained in the FCS or interfering with downstream EV analysis. This statement is based on side–by–side observations, performed in our laboratory, on cell proliferation rate of ASCs cultured both under normoxic and hypoxic conditions, with particle–depleted FCS or untreated FCS.

It is generally not reported whether, non properly vesicle–depleted serum may bias downstream applications regarding EV isolation, analysis and functional translation.[66, 67, 71] Cvjetkovic et al., in their study, include in their cell cultures and, consequently, harvest in the conditioned medium also the suspension phase of purified bovine serum. This implies the inclusion of a big number of contaminating vesicles among the precipitated samples that may alter, for instance, the quality of the purified mRNAs. The lack of information on the characteristics of animal–derived vesicles may bias the interpretation of the obtained results.

In the present study, we present an easily reproducible and highly efficient method in order to produce virtually particle–free FCS. To allow efficient elimination of vesicles, and due to the high viscosity of serum, it is recommended to centrifuge serum diluted to at most 10% in the appropriate culture medium.

6.2 Cell Death & Microbial Contamination

Assessment of cell viability was performed by Trypan blue exclusion and the maximum acceptable cell death percentage in culture was arbitrarily set to 5%. This provided reasonably pure EV release by live cells. Microbial contamination was qualitatively assessed both by optical and electron microscopy, confirming pure from contamination exosomal fractions.

Dying or dead cells release vesicles of various sizes, that can eventually break into smaller fragments upon ultracentrifugation or filtration. Thus, the presence of abundant dead cells can, eventually, lead to contamination of viable cell–derived EVs by apoptotic vesicles. These vesicles cannot be separated with the current purification protocols and will consequently alter the protein and/or nucleic acid composition of the isolated EV fractions.[87–89] For the same reasons, bacterial, mycoplasma or even viral contamination can radically alter the quality of the isolated particles. Whatever the culture conditions, it is absolutely necessary to quantify the percentage of dead cells present in the culture, as well as to ensure that no microbial contamination has occurred at the end of the conditioning period.

6.3 Ultracentrifugation & Polymeric Precipitation

In the present investigation,sequential ultracentrifugation allowed to isolate particles with an ample size distribution and characteristics, as assessed by NTA, that assemble a vesicular population which overlaps that of both exosomes and microvesicles. Due to significantly, compared to kit–isolated particles, low concentration it was not possible to either visualize (by TEM/IEM and AFM) or identify any exosomes (by EV Array).

Combination of differential centrifugation, sample concentration and a commercially available polymeric precipitation reagent resulted in high quality isolated exosomes. Overnight incubation with the precipitation reagent, low–speed centrifugation and decontamination of the sample from unbound dye, resulted in highly enriched exosome fractions. Although, the input volume was identical for the the two isolation techniques, the particle yield by polymeric precipitation, was significantly higher than the ultracentrifugated samples. The relative particle concentrations were assessed by NTA and micro–BCA assay and the results are consistent with several investigations involved in comparing the aforementioned isolation methods.[71, 72, 90, 91] The work done both by King et al. and Rekker et al., for instance, confirm the advantages presented by polymeric precipitation compared to ultracentrifugation, in terms of exosome yield and recovery of specific proteins and micro RNAs from the particles isolated.

Atomic force microscopy and electron microscopy confirmed the presence of exosomes in the kit–isolated samples and phenotypical characterization by EV array validated the presence of surface proteins reportedly associated with exosomes (CD9, CD63 and CD81).[92–94]

Electron microscopy revealed the presence of contaminating lipoproteins and microsomes in the isolated samples, observations that are consistent with other investigations, where it is shown that both polymer precipitation and ultracentrifugation have a tendency to include numerous non–vesicular contaminants.[95]

It is clear, from the results presented in the present study, that polymeric precipitation is superior to ultracentrifugation in isolating exosomes. It is, however, highly recommended, before proceeding with the particle isolation, to thoroughly pre–clear and concentrate the samples in order to increase the efficiency of the adopted protocol.

6.4 Adipose–Derived Stem Cells & Hypoxia

In the present study, in order to assess exosome production and release under hypoxic conditions, cells from four different ASC batches were cultured at 1% of oxygen concentration. No significant differences, regarding exosome release, particle morphology and phenotype, were observed among the different cell batches expanded under normoxic (20% O_2) and hypoxic conditions (1% O_2). In contrast to other investigations, where hypoxic conditioning enhances the release of EVs, exosome release in the presence of 1% oxygen was slightly decreased, compared to normoxic conditioned cultures. King et al. in their work on breast cancer cells, revealed significantly more nanoparticles isolated under hypoxic conditions (both 1% and 0.1% of O_2 concentration) relative to normoxic controls.[71, 77, 78]

However, this effect could be explained by the physiological differences between the different cell lines under examination and further investigation with different oxygen concentrations is necessary to fully comprehend the effect of low–oxygen–concentration on ASC–derived–exosome production and release.

6.5 The Effect on Exosomal Yield by Prolonged Culturing Periods

Investigating the effect of oxygen concentration on EV release, lead to the observation that the relation between the amount of cells present in the culture flasks at the moment of conditioned medium harvesting, and the relative isolated particles was proportionally inverse. Current *in vitro* investigations intent on assessing EVs from cell culture supernatants generally suggest to expand the cells until they reach around 80% confluence

(in case of adherent cells).[12, 68]
The results confirmed the hypothesis, that prolonged culturing has negative impact on exosome release, resulting in a lower yield of isolated particles. This effect may be due to the fact that exosomes are released to the extracellular compartment by fusion of the MVBs with the delimiting plasma membrane, and high cell density could hinder this process. This effect could be limited to the cell lines under examination in the present study and although, no other reports relating cell density to particle release are available, side–by–side comparisons with different cell lines are necessary in order to comprehend the phenomenon.

6.6 Believe in What You See

In the present study, kit–isolated particles were assessed by TEM and IEM, techniques that confirmed the presence of particles in the isolated fractions, which size, morphology and surface markers correspond to that of the exosomes.
With the aid of atomic force microscopy and it's possibility of sub–nanometer resolution it was able to visualize particles which morphology and size correspond to that of the exosomes, thus confirming, previously performed, electron microscopy particle assessment. This results are in line with numerous publications where AFM has been used to study extracellular vesicles.[73, 80, 81]
Western blotting may be used to detect the presence of specific surface proteins reportedly associated with extracellular vesicles or EV subpopulations such as the tetraspanins CD9, CD63 and CD81. This technique has, however, some limitations as it is not suitable for quantification assays, it requires large amounts of purified vesicles and on its own cannot identify whether proteins are from EVs.[54, 74]
In the present study, in order to characterize the EV fractions isolated by polymeric precipitation, a highly sensitive extracellular vesicle array (EV Array) was employed.[86] EV array enabled the detection and phenotypical characterization of exosomes both from unpurified starting material and purified exosomal fractions in a high–throughput manner. The antibodies used for that purpose (CD9, CD63 and CD81), ensured that all exosomes captured were detected, as well as other types of vesicles were excluded from detection. No substantial differences were observed in the aforementioned surface markers expression, among kit–isolated particles derived from both normoxic and hypoxic conditioned medium. However, there was a two fold increase in the expression of the same markers when untreated samples were compared to kit–purified exosomal fractions. This confirms the high level of detection sensitivity displayed by the EV Array as well as the ability of the polymer–based exosome isolation to produce high yield of specific particles. A comparison of the exosomal fractions isolated by ultracentrifugation and

polymeric precipitation, did not produce any interpretable results as the input volume of ultracentrifugated vesicles was below EV arrays limit of detection and no signal was registered.
It is important to emphasize that both NTA, transmission electron microscopy, AFM and EV array were indispensable tools in validating the cell culture techniques and particle–isolation protocols adopted in the present investigation and underline the necessity to adopt the aforementioned EV characterization methods as standard practices in EV study.

6.7 Concluding Remarks & Perspectives

In the present study it is highlighted the need for standardization of cell culture procedures, particle isolation and analysis techniques in order to facilitate comparison of results and achieving consensus in EV study.
We managed to successfully produce virtually particle–free FCS, important constituent of the environment where cells are expanded, thus avoiding possible bias in downstream EV analysis given by animal–origin contaminating particles.
Although the "gold standard" in exosome isolation is sequential ultracentrifugation, it proven unable to produce sufficient EVs that could enable their characterization and further analysis. Combination of differential centrifugation, concentration and polymeric precipitation, on the other hand, resulted in high quality small EV isolation. However, further investigation and optimization of the former isolation method is mandatory in order to produce comparable results.
In the current study, hypoxia did not have any substantial effect on exosome morphology and yield. However, further investigation is needed in order to comprehend the effect of different than 1% oxygen concentration both on the amount and the quality of particles released under hypoxic conditions.
Another important aspect of standard cell culturing, as displayed in the current project is the relation between cell density and exosome release. The close relation between these two factors emphasizes the necessity to harvest conditioned culture medium while cells are still in low confluency, in order to maximize exosome yield.
The results obtained in the present study, both regarding the definition of optimal cell culture conditions, isolation and exosome characterization protocols, pave the path for *in vitro* and *in vivo* functional translation, of the regenerative properties comprised in the different fractions of the ASC secretome.

Appendix A

AFM High Resolution Images

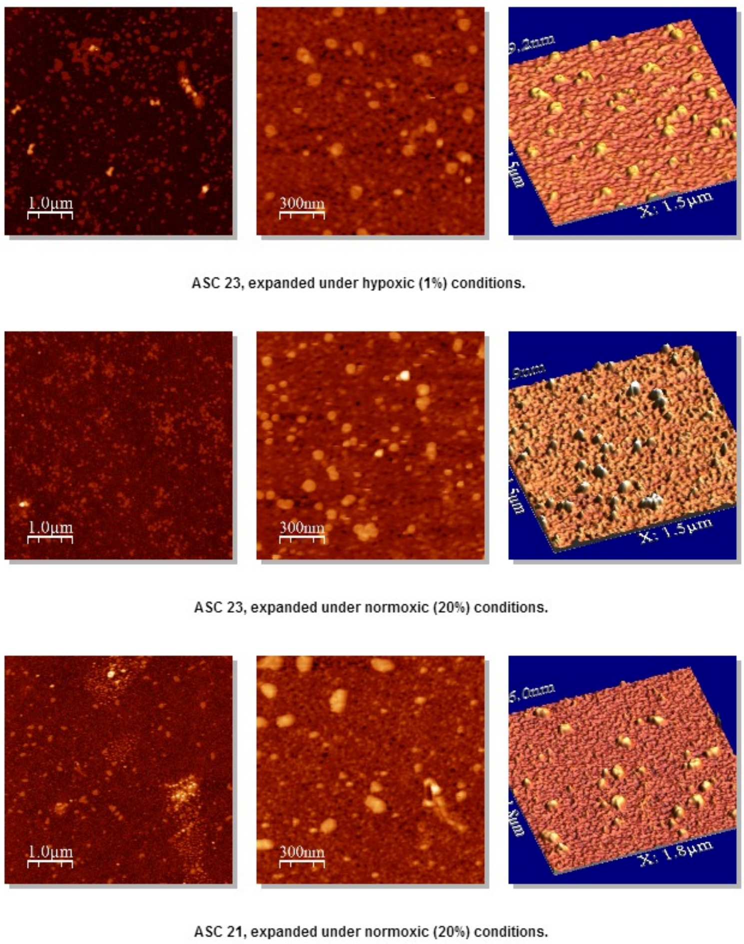

FIGURE A.1: AFM images of exosomes isolated from two different cell lines, conditioned under normoxia (20% oxygen) and hypoxia (1% oxygen), on mica substrate with 3D topography inserted.

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