Cardioprotective and proangiogenic activities of small extracellular vesicles released by amniotic fluid stem cells

Thesis submitted by

Kaloyan Valentin Takov
BSc (First class with Honours) MRSB

For the degree of Doctor of Philosophy

University College London
Institute of Cardiovascular Science
The Hatter Cardiovascular Institute
67 Chenies Mews
London WC1E 6HX
United Kingdom
September 2019
Declaration

I, Kaloyan Valentin Takov, confirm that the work presented in this thesis is my own. I confirm that information derived from other sources has been properly acknowledged in the text. All the collaborations and experimental assistance is also duly acknowledged.
Acknowledgements

I express my sincere gratitude to my supervisors, Prof Sean Davidson and Prof Derek Yellon. Prof Davidson was always promptly responding to any questions or doubts I might have had, and his advice was indispensable for the research performed during this project. Prof Yellon’s advice and directions were always most helpful. Their enthusiasm and passion for science inspired me in good and bad times. Prof Davidson and Prof Yellon’s guidance extended beyond the experimental research presented here: their support for conference presentations and award applications as well as provision of general scientific guidance was invaluable.

I would also like to thank the whole team of the Hatter Cardiovascular Institute for their help. In particular, I am grateful to Dr Zhenhe He who performed the in vivo ischaemia/reperfusion experiments. I further acknowledge the generous help and moral support provided by Dr Jaime Riquelme Melendez. I thank Dr Sapna Arjun, Dr Catherine Wilder and Dr Staša Taferner for creating a friendly and pleasant laboratory environment to work in.

I extend my thanks to Dr Pascale Guillot for kindly providing the amniotic fluid stem cells used for a big portion of the research presented here. I am also grateful to Dr Filipa Vlahova for her introductory guidance on the work with these cells and her time spent in endless discussions.

I would like to thank Dr John Timms and Dr Harvey Johnston for performing the mass spectrometry experiments and for their advice on experimental design and data interpretation. I am also thankful for the assistance I received from Mark Turmaine with the electron microscopy.

Finally, I am sincerely grateful to my parents and Aneliya, my other half, who were always there for me when I needed them in the past 4 years, and whose support in this endeavour of mine was immeasurable.
Publication list

Articles


Abstracts

Abstract

Protection against myocardial ischaemia/reperfusion injury and regeneration of the damaged myocardium are long-sought goals. The use of small extracellular vesicles (sEVs) released by mesenchymal stem cells (MSCs) was shown to be of benefit in the myocardial infarction setting. However, MSCs are frequently harvested from aged or diseased patients and suboptimal sEV isolation methods are used. A subtype of young, foetal MSCs, namely spindle-shaped amniotic fluid stem cells (SS-AFSCs), is known to possess better expansion and functional capacity than its adult counterparts. Here, sEVs released by SS-AFSCs were isolated using size-exclusion chromatography (SEC) – an isolation technique that yields vesicles of superior purity – and their cardioprotective and proangiogenic activities were studied.

Firstly, using rat blood plasma, it was demonstrated that SEC isolates higher sEV yields with significantly compromised purity, mostly due to the presence of lipoproteins. To overcome this, a serum-free environment was used for sEVs isolation from SS-AFSC-conditioned medium. Comprehensive characterisation experiments showed that the harvested SS-AFSC sEVs are of high purity. Functionally, SS-AFSC sEVs protected the rat myocardium from ischaemia/reperfusion injury in vivo, but not isolated cardiomyocytes in vitro, indicative of indirect cardioprotective effects. Additionally, SS-AFSC sEVs promoted migration of endothelial cells in vitro and recapitulated the promigratory effects of the SS-AFSC-conditioned medium. Using pharmacological inhibition, it was shown that PI3K pathway, a known player in cell migration, mediates the sEV effects, while a series of potential candidates in the sEV cargo were excluded. Finally, cellular sEV uptake was studied by use of lipophilic dye-labelling experiments. Surprisingly, this commonly used approach was found to be unsuitable for sEV tracking due to non-specific dye retention by non-sEV contaminants.

Overall, SEC-isolated SS-AFSC sEVs possess cardioprotective potential manifested only in vivo, and promigratory activity which requires PI3K signalling. These data indicate that SS-AFSC sEVs have multifactorial beneficial effects in a myocardial infarction setting.
Impact Statement

The aim of the research presented in this thesis is to achieve better and safer treatments for patients who have suffered a myocardial infarction – one of the biggest causes of morbidity and mortality. Protection of the heart from the injury that occurs during and after a myocardial infarction, as well as repair and regeneration of this vital organ are long-sought goals. It has recently been discovered that stem cells and their secretome can be beneficial in this setting. Furthermore, it has been shown that the benefits of the stem cells are mainly delivered by secreted small extracellular vesicles (sEVs, or “exosomes”). However, prior to translating this research into clinic, further mechanistic understanding of the benefits provided by stem cell sEVs is necessary. Moreover, the procedures used to obtain sEVs and to study their interactions with other cells are still being optimised, and duly characterised sEV isolates are required for successful clinical application.

Size-exclusion chromatography (SEC) is a method that has been increasingly recognised as one of the best for sEV purification. However, various limitations and insufficient characterisation of the obtained sEVs preclude complete implementation of this technique. Here, SEC was characterised as a method for sEV isolation of blood plasma or stem cell-conditioned culture medium. SEC full potential was only realised in an environment devoid of blood products, mainly due to interference of blood-derived lipoproteins with the process. The findings of this initial characterisation were published and are expected to influence the method of choice in future experiments with plasma samples and can also have great clinical impact in studies for discovery of biomarkers for different pathologies.

Additionally, SEC was used to harvest sEVs from a potent foetal stem cell type, namely amniotic fluid stem cells (AFSCs). AFSCs have superior characteristics to most adult mesenchymal stem cells which makes them an attractive potential treatment for patients with myocardial infarction. The limitations of SEC were overcome by using culture conditions devoid of blood products, thus isolating high yields of pure AFSC sEVs. This study demonstrated that in the setting of myocardial infarction AFSC sEVs are cardioprotective when used as an intravenous injection. Importantly, this is a
clinically applicable administration with translational potential. It was further demonstrated here that AFSC sEVs potently stimulate migration of endothelial cells and various mechanistic studies to identify the players involved were performed. These data open new avenues for further exploration of AFSCs as a potent novel cardioprotective source. The impact of these findings has been recognised with a prize at an international conference.

Finally, research on experimental tools to label sEVs and track their fate is of extreme importance. Here, a commonly used approach – sEV labelling with lipophilic dyes – was shown to lead to marked artefactual staining due to the non-specific nature of the dyes. These findings have been published and their importance is already evidenced by the high citation record of this publication. Furthermore, this article is motivating researchers to seek new tools for sEV labelling and improve the existing ones.
Contents

Declaration ............................................................................................................................................... 2
Acknowledgements .............................................................................................................................. 3
Publication list ...................................................................................................................................... 4
Abstract ........................................................................................................................................... 5
Impact statement ............................................................................................................................... 6
Contents ........................................................................................................................................ 8
List of tables ....................................................................................................................................... 13
List of figures ..................................................................................................................................... 14
List of key acronyms .......................................................................................................................... 16

Chapter 1. Background ................................................................................................................... 18
  1.1. Coronary artery disease (CAD) ............................................................................................... 18
  1.2. Myocardial infarction ............................................................................................................. 18
  1.3. Ischaemia/reperfusion injury and cardioprotection ............................................................... 19
  1.4. The present and future of cardioprotection ......................................................................... 21
  1.5. Angiogenesis for cardioprotection and long-term benefits ................................................... 23
  1.6. Extracellular vesicles ........................................................................................................... 25
      1.6.1. Exosomes as major components of the sEV population: formation and release .......... 28
      1.6.2. sEV isolation and purity ................................................................................................. 29
      1.6.3. sEVs and cardioprotection ............................................................................................. 35
  1.7. Amniotic fluid stem cells ....................................................................................................... 42

Chapter 2. Materials and methods ................................................................................................. 45
  2.1. Animals, cells and reagents ................................................................................................... 45
      2.1.1. Animals and reagents ................................................................................................... 45
      2.1.2. Cell culture .................................................................................................................... 46
      2.1.2.1. Mouse cardiac endothelial cells (MCECs) ................................................................. 46
      2.1.2.2. Human umbilical vein endothelial cells (HUVECs) .................................................. 46
      2.1.2.3. Spindle-shaped amniotic fluid stem cells (SS-AFSCs) ................................................. 46
      2.1.2.4. Isolation and culture of primary adult rat ventricular cardiomyocytes (ARVCs) ........ 47
  2.2. Isolation of sEVs .................................................................................................................... 48
      2.2.1. Isolation of sEVs from rat blood ....................................................................................... 48
      2.2.1.1. Preparation of plasma for ultracentrifugation (UC) or size-exclusion chromatography (SEC) .......................................................................................................................... 48
      2.2.1.2. Isolation of plasma sEVs using UC ............................................................................... 48
      2.2.1.3. Isolation of plasma sEVs using SEC ............................................................................ 49
      2.2.2. Isolation of sEVs from cell-conditioned culture medium ................................................ 50
      2.2.2.1. Preparation of conditioned medium for SEC ................................................................. 50
2.2.2.2. Isolation of sEVs using SEC ................................................................. 50

2.3. CHARACTERISATION OF THE ISOLATED SEVs ........................................... 52
  2.3.1. Nanoparticle tracking analysis (NTA) ......................................................... 52
  2.3.2. Protein content ......................................................................................... 52
  2.3.3. Dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) ... 53
  2.3.4. Transmission electron microscopy (TEM) .................................................. 54
  2.3.5. Dot blot protein analysis ............................................................................. 54
  2.3.6. Proteomics: liquid chromatography – tandem mass spectrometry (LC-MS/MS) ... 55
  2.3.7. Triglyceride content .................................................................................. 56
  2.3.8. EV-TRACK .............................................................................................. 57

2.4. SEV UPTAKE STUDIES .................................................................................. 57

2.5. IN VITRO MODELS OF REACTIVE OXYGEN SPECIES- AND HYPOXIA/REOXYGENATION-INDUCED CELL DEATH . 57
  2.5.1. Reactive oxygen species (ROS)-induced cell death .......................................... 57
  2.5.2. Hypoxia/reoxygenation-induced cell death (simulated IRI in vitro) .................. 58
    2.5.2.1. Cells and composition of the buffers ......................................................... 58
    2.5.2.2. Experimental procedure ......................................................................... 59
  2.5.3. Lactate dehydrogenase (LDH) assay .......................................................... 59

2.6. IN VIVO MODEL OF NON-RECOVERY IRI IN RATS ...................................... 60
  2.6.1. Anaesthesia, ventilation and preparation for the procedure ......................... 60
  2.6.2. Induction of myocardial infarction .............................................................. 60
  2.6.3. Heart collection and staining .................................................................... 62
  2.6.4. Area-at-risk (AAR) and infarct size (IS) measurements .............................. 62

2.7. ENDOTHELIAL CELL MIGRATION ASSAYS ................................................. 64

2.8. ENDOTHELIAL CELL PROLIFERATION ASSAYS ......................................... 66

2.9. ENDOTHELIAL CELL TUBE FORMATION ASSAYS ....................................... 66

2.10. FLOW CYTOMETRY ....................................................................................... 67

2.11. WESTERN BLOTTING ................................................................................. 68
  2.11.1. Conventional Western blotting ................................................................. 68
  2.11.2. Wes™ Simple Western ........................................................................... 69

2.12. PROTEIN ARRAYS ..................................................................................... 69

2.13. STATISTICAL ANALYSIS AND DISPLAY ITEMS ......................................... 70

Chapter 3. Comparison of SEC and UC for blood plasma sEV isolation .............. 71

3.1. INTRODUCTION ............................................................................................ 71

3.2. AIMS .............................................................................................................. 73

3.3. METHODS ..................................................................................................... 74
  3.3.1. Blood plasma sEV isolation ....................................................................... 74
  3.3.2. Protein content ........................................................................................ 74
  3.3.3. Dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) .... 74
3.3.4. Wes™ Simple Western

3.4. RESULTS

3.4.1. Protein and particle content of sEV samples isolated by SEC or UC of blood plasma

3.4.2. Yield and purity of the sEV samples obtained by SEC or UC of blood plasma

3.5. DISCUSSION

Chapter 4. Isolation and characterisation of SS-AFSC sEVs

4.1. INTRODUCTION

4.2. AIMS

4.3. METHODS

4.3.1. SS-AFSC serum-free culture

4.3.2. Flow cytometry

4.3.3. SS-AFSC sEV isolation

4.3.4. Nanoparticle tracking analysis (NTA)

4.3.5. Protein content

4.3.6. Dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA)

4.3.7. Dot blot protein analysis

4.3.8. Proteomics

4.4. RESULTS

4.4.1. SS-AFSC characterisation after 24 h or 48 h of serum-free culture

4.4.2. Isolation of SS-AFSC sEVs after 24 h or 48 h of serum-free culture of SS-AFSCs

4.4.3. Comprehensive characterisation of SS-AFSC sEVs

4.5. DISCUSSION

Chapter 5. Cardioprotective effects of SS-AFSC sEVs in vitro and in vivo

5.1. INTRODUCTION

5.2. AIMS

5.3. METHODS

5.3.1. ROS-induced cell death

5.3.2. Hypoxia/reoxygenation-induced cell death (simulated IRI in vitro)

5.3.3. In vivo model of non-recovery IRI in rats

5.3.4. Proteomics

5.4. RESULTS

5.4.1. Cardioprotective effects of SS-AFSC sEVs in vitro: direct protection of isolated cardiomyocytes

5.4.2. Cardioprotective effects of SS-AFSC sEVs in vivo

5.4.3. Cardioprotective effects of SS-AFSC sEVs in vitro: indirect protection of isolated cardiomyocytes by effects on endothelial cells

5.5. DISCUSSION
Chapter 6. Proangiogenic effects of SS-AFSC sEVs in vitro ........................................ 141

6.1. INTRODUCTION ........................................................................................................... 141
6.2. AIMS ............................................................................................................................. 142
6.3. METHODS ...................................................................................................................... 143
  6.3.1. Endothelial cell migration assays ............................................................................. 143
  6.3.2. Endothelial cell proliferation assays ........................................................................ 144
  6.3.3. Endothelial cell tube formation assays .................................................................... 144
  6.3.4. Protein content ....................................................................................................... 145
6.4. RESULTS ....................................................................................................................... 146
  6.4.1. SS-AFSC sEV effects on endothelial cell migration, proliferation and tube formation in vitro .................................................................................................................................... 146
  6.4.2. Comparison of SS-AFSC sEV and starting conditioned medium effects on endothelial cell migration in vitro ............................................................................................................ 149
6.5. DISCUSSION .................................................................................................................. 153

Chapter 7. SS-AFSC sEV cargo and the mechanisms of the promigratory effects of the sEVs ................................................................. 153

7.1. INTRODUCTION ............................................................................................................. 157
7.2. AIMS ............................................................................................................................. 159
7.3. METHODS ...................................................................................................................... 161
  7.3.1. Protein arrays ........................................................................................................... 161
  7.3.2. Proteomics ............................................................................................................... 162
  7.3.3. Endothelial cell migration ....................................................................................... 162
  7.3.4. Western blotting ..................................................................................................... 163
7.4. RESULTS ....................................................................................................................... 164
  7.4.1. SS-AFSC sEV cargo .............................................................................................. 164
  7.4.2. SS-AFSC sEV promigratory effects: mediators in the SS-AFSC sEV cargo ............ 172
  7.4.3. SS-AFSC sEV promigratory effects: signalling effectors in the target endothelial cells .................................................................................................................................... 174
7.5. DISCUSSION .................................................................................................................. 177

Chapter 8. Lipophilic dye labelling as a means of studying the mechanisms of SS-AFSC sEV effects ................................................................. 182

8.1. INTRODUCTION ............................................................................................................. 182
8.2. AIMS ............................................................................................................................. 183
8.3. METHODS ...................................................................................................................... 184
  8.3.1. Materials ................................................................................................................. 184
  8.3.2. sEV isolation ........................................................................................................... 184
  8.3.3. Nanoparticle tracking analysis (NTA) ..................................................................... 184
  8.3.4. Protein content ....................................................................................................... 185
8.3.5. Dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) ......................... 185
8.3.6. Triglyceride content ........................................................................................................ 185
8.3.7. sEV uptake .................................................................................................................... 186
  8.3.7.1. Sample labelling procedure ....................................................................................... 186
  8.3.7.2. Cellular uptake of labelled samples .......................................................................... 187
8.4. RESULTS ........................................................................................................................... 188
  8.4.1. Particle, protein, sEV and lipoprotein content of SEC fractions of blood plasma ...... 188
  8.4.2. Interference of contaminants with lipophilic dye labelling of blood plasma sEVs ...... 190
  8.4.3. Interference of contaminants with lipophilic dye labelling of SS-AFSC sEVs ......... 192
  8.4.4. Lipophilic dye labelling of serum and pure protein samples as a means of investigating contaminant artefacts in uptake experiments ......................................................... 196
8.5. DISCUSSION .................................................................................................................... 201

Chapter 9. Summary and outlook ......................................................................................... 206
  9.1. SUMMARY OF FINDINGS .............................................................................................. 206
  9.2. OUTLOOK AND FURTHER STUDIES ........................................................................... 207

Chapter 10. References ......................................................................................................... 211
List of tables

**TABLE 1-1.** OVERVIEW OF SOME OF THE MOST COMMONLY USED METHODS FOR SEV ISOLATION .......................................................... 31

**TABLE 2-1.** COMPOSITION OF NORMOXIC AND HYPOXIC BUFFERS USED IN SIMULATED IRI EXPERIMENTS *IN VITRO* .......................... 58

**TABLE 3-1.** ANTIBODIES USED FOR DELFIA STUDIES ................................................................................................................. 75

**TABLE 4-1.** ANTIBODIES USED FOR SS-AFSC CHARACTERISATION BY FLOW CYTOMETRY ........................................................ 93

**TABLE 4-2.** ANTIBODIES USED FOR DELFIA STUDIES .................................................................................................................... 95

**TABLE 4-3.** BOVINE PROTEINS DETECTED IN THE SS-AFSC-CONDITIONED MEDIUM OR THE SEC-ISOLATED SS-AFSC sEVs
(SEE THE NEXT PAGE) ........................................................................................................................................................................... 107

**TABLE 5-1.** PROTEINS ASSOCIATED WITH THE GENE ONTOLOGY BIOLOGICAL PROCESS TERM “NEGATIVE REGULATION OF CELL DEATH” IN SS-AFSC SEV SAMPLES (SEE THE NEXT PAGE) ......................................................................................................................... 129

**TABLE 7-1.** ANTIBODIES USED FOR WESTERN BLOTTING ANALYSIS OF SIGNALLING PATHWAYS IN HUVECs ......................... 163

**TABLE 8-1.** ANTIBODIES USED FOR DELFIA STUDIES .................................................................................................................... 185
List of figures

FIGURE 1-1. MYOCARDIUM SALVAGEABLE BY REPERFUSION AS A FUNCTION OF THE DURATION OF ISCHAEMIA .......... 20
FIGURE 1-2. ENDOSOMAL SEV SECRETION AND MORPHOLOGY ........................................................................ 27
FIGURE 1-3. SIZE-DENSITY PLOT OF SEVs AND COMMON SEV CONTAMINANTS ............................................ 30
FIGURE 1-4. MECHANISMS OF THE CARDIOPROTECTIVE AND REGENERATIVE BENEFITS CONFERRED BY MESENCHYMAL
STEM/STROMAL CELLS (MSCs) ...................................................................................................................... 38
FIGURE 2-1. PRINCIPLE OF SIZE-EXCLUSION CHROMATOGRAPHY (SEC) FOR ISOLATION OF SEVs ...................... 49
FIGURE 2-2. ISOLATION OF SEVs FROM CONDITIONED CELL CULTURE MEDIUM USING SEC .............................. 51
FIGURE 2-3. ECG CHANGES DURING IN VIVO ISCHAEMIA/REPERFUSION IN RATS .............................................. 61
FIGURE 2-4. IN VIVO MODEL OF NON-RECOVERY IRI IN RATS ........................................................................ 63
FIGURE 2-5. MODIFIED BOYDEN’S CHAMBER MIGRATION ASSAY SETUP AND ANALYSIS .............................. 65
FIGURE 3-1. PILOT DATA FROM SEC FRACTIONATION OF RAT BLOOD PLASMA .............................................. 77
FIGURE 3-2. PROTEIN CONTENT AND PARTICLE NUMBERS OF SEV ISOLATES OF RAT BLOOD PLASMA SUBJECTED TO SEC OR
UC ............................................................................................................................................................. 78
FIGURE 3-3. SEV-SPECIFIC MARKERS IN SAMPLES OF RAT BLOOD PLASMA SUBJECTED TO SEC OR UC ................. 81
FIGURE 3-4. APOB, SEV MARKERS AND MEDIUM AND LARGE EV MARKERS IN THE SEV ISOLATES OF RAT BLOOD PLASMA
SUBJECTED TO SEC OR UC ......................................................................................................................... 82
FIGURE 3-5. TEM IMAGES OF SEV ISOLATES OF RAT BLOOD PLASMA SUBJECTED TO SEC OR UC ..................... 83
FIGURE 3-6. YIELD AND PURITY OF SEV ISOLATES OF RAT BLOOD PLASMA SUBJECTED TO SEC OR UC ............... 84
FIGURE 4-1. CHARACTERISATION OF SS-AFSCs BY FLOW CYTOMETRY ............................................................ 97
FIGURE 4-2. NUMBERS AND CELL DEATH OF SS-AFSCs INCUBATED WITH OR WITHOUT SERUM ........................ 98
FIGURE 4-3. SS-AFSC EXPRESSION OF MEMBRANE MARKERS AFTER INCUBATION WITH OR WITHOUT SERUM....... 99
FIGURE 4-4. ISOLATION OF SS-AFSC SEVs USING SEC AND CHARACTERISATION OF THE ISOLATES (SEE THE PREVIOUS
PAGE) ......................................................................................................................................................... 102
FIGURE 4-5. CHARACTERISATION OF POOLED SEV-RICH SS-AFSC SEC FRACTIONS: PARTICLE NUMBER, PROTEIN CONTENT
AND PARTICLE RECOVERY .......................................................................................................................... 104
FIGURE 4-6. CHARACTERISATION OF POOLED SEV-RICH SS-AFSC SEC FRACTIONS: SEV-SPECIFIC MARKER CONTENT,
MORPHOLOGY AND CARGO .......................................................................................................................... 106
FIGURE 5-1. EXPERIMENTAL DESIGN: ESTABLISHMENT OF AN H2O2-INDUCED CELL DEATH MODEL .................. 118
FIGURE 5-2. EXPERIMENTAL DESIGN: SS-AFSC SEV EFFECTS IN THE H2O2-INDUCED CELL DEATH MODEL ........... 119
FIGURE 5-3. EXPERIMENTAL DESIGN: SIMULATED IRI MODEL IN VITRO (FIRST EXPERIMENT) ............................. 121
FIGURE 5-4. EXPERIMENTAL DESIGN: ENDOTHELIAL CELL-MEDIATED PROTECTION OF CARDIomyOCyTES IN THE SIMULATED
IRI MODEL IN VITRO (SECOND EXPERIMENT) .............................................................................................. 122
FIGURE 5-5. EXPERIMENTAL DESIGN: NON-RECOVERY IRI IN RATS IN VIVO .................................................... 124
FIGURE 5-6. ESTABLISHMENT OF A MODEL OF H2O2-INDUCED ARVC DEATH .................................................. 127
FIGURE 5-7. EFFECTS OF SS-AFSC SEVs ON H2O2-INDUCED ARVC DEATH .................................................... 128
FIGURE 5-8. EFFECTS OF SS-AFSC SEVs ON SIMULATED IRI IN VITRO ............................................................... 129
FIGURE 5-9. EFFECTS OF SS-AFSC sEVs ON IRI IN RATS IN VIVO .................................................. 132
FIGURE 5-10. IRI IN VIVO: ANIMAL WEIGHTS AND VARIABILITY OF THE ANALYSES ...................... 133
FIGURE 5-11. EFFECTS OF THE STABLE sEV-TREATED HUVEC SECRETOME ON SIMULATED IRI IN VITRO .................................................................................................................... 135
FIGURE 6-1. ESTABLISHMENT OF A MODIFIED BOYDEN'S CHAMBER ASSAY OF ENDOTHELIAL CELL MIGRATION ................................................................. 147
FIGURE 6-2. EFFECTS OF SS-AFSC sEVs IN MODELS OF ANGIOGENESIS IN VITRO (SEE THE PREVIOUS PAGE) ......................................................................................... 149
FIGURE 6-3. COMPARISON OF THE PROMIGRATORY EFFECTS OF STARTING SS-AFSC-CONDITIONED MEDIUM AND ISOLATED sEVs ........................................................................................................... 151
FIGURE 7-1. CYTOKINE AND ANGIOGENIC FACTOR CARGO OF SS-AFSC-CONDITIONED MEDIUM AND SS-AFSC sEVs ..................................................................................... 166
FIGURE 7-2. PROTEINS IN SS-AFSC-CONDITIONED MEDIUM AND SS-AFSC sEVs ................................................................................................................................. 167
FIGURE 7-3. GENE ONTOLOGY (GO) BIOLOGICAL PROCESS TERMS OVERREPRESENTED IN SS-AFSC sEVs ............................................................................................... 169
FIGURE 7-4. STRING INTERACTION NETWORK OF PROTEINS ENRICHED IN OR EXCLUSIVE TO THE SS-AFSC sEVs .................................................................................. 170
FIGURE 7-5. KEGG PATHWAYS OVERREPRESENTED IN SS-AFSC sEVs ................................................................................................................................. 171
FIGURE 7-6. MECHANISM OF SS-AFSC sEV-INDUCED ENDOTHELIAL CELL MIGRATION: MEDIATORS IN THE sEV CARGO .................................................................................................................. 173
FIGURE 7-7. MECHANISM OF SS-AFSC sEV-INDUCED ENDOTHELIAL CELL MIGRATION: INVOLVEMENT OF ENDOTHELIAL PI3K PATHWAY .................................................................................................................. 175
FIGURE 7-8. MECHANISM OF SS-AFSC sEV-INDUCED ENDOTHELIAL CELL MIGRATION: sEV-INDUCED ACTIVATION OF KINASES IN THE ENDOTHELIAL CELLS .................................................................................................................. 176
FIGURE 8-1. CHARACTERISATION OF SEC-FRACTIONATED RAT BLOOD PLASMA (SEE THE PREVIOUS PAGE) ................................................................................................. 190
FIGURE 8-2. UPTAKE OF PLASMA SEC FRACTIONS INTO ENDOTHELIAL CELLS AND CARDIOMYOCYTES (SEE THE PREVIOUS PAGE) .................................................................................................................. 192
FIGURE 8-3. CHARACTERISATION OF SEC FRACTIONS FROM SS-AFSC-CONDITIONED MEDIUM AND THEIR ENDOTHELIAL CELL UPTAKE .................................................................................................................. 194
FIGURE 8-4. IMPACT OF THE CHOICE OF LIPOPHILIC DYE ON THE UPTAKE OF SEC FRACTIONS FROM SS-AFSC-CONDITIONED MEDIUM IN ENDOTHELIAL CELLS .................................................................................................................. 195
FIGURE 8-5. UPTAKE OF LABELLED SERUM AND PURE PROTEIN SAMPLES IN ENDOTHELIAL CELLS AND CARDIOMYOCYTES (SEE THE PREVIOUS PAGE) .................................................................................................................. 199
FIGURE 8-6. UPTAKE OF SERUM AND UC-DERIVED SERUM FRACTIONS IN ENDOTHELIAL CELLS .................................................................................................................. 200
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAR</td>
<td>Area at risk</td>
</tr>
<tr>
<td>AFSC(s)</td>
<td>Amniotic fluid stem cell(s)</td>
</tr>
<tr>
<td>AGO</td>
<td>Argonaute protein</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APOB</td>
<td>Apolipoprotein B</td>
</tr>
<tr>
<td>ARVC</td>
<td>Adult rat ventricular cardiomyocytes</td>
</tr>
<tr>
<td>AU</td>
<td>Arbitrary units</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BGN</td>
<td>Biglycan</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CDC</td>
<td>Cardiosphere-derived cells</td>
</tr>
<tr>
<td>CM</td>
<td>Conditioned medium</td>
</tr>
<tr>
<td>CPC</td>
<td>Cardiac progenitor cells</td>
</tr>
<tr>
<td>DELFIA</td>
<td>Dissociation-enhanced lanthanide fluorescence immunoassay</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>EV(s)</td>
<td>Extracellular vesicle(s)</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>GMP</td>
<td>Good Manufacturing Practice</td>
</tr>
<tr>
<td>GO</td>
<td>Gene Ontology</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>HUVEC(s)</td>
<td>Human umbilical vein endothelial cell(s)</td>
</tr>
<tr>
<td>IDL</td>
<td>Intermediate-density lipoprotein</td>
</tr>
<tr>
<td>IPC</td>
<td>Ischaemic preconditioning</td>
</tr>
<tr>
<td>IRI</td>
<td>Ischaemia-reperfusion injury</td>
</tr>
<tr>
<td>IS</td>
<td>Infarct size</td>
</tr>
<tr>
<td>LAD artery</td>
<td>Left anterior descending artery</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography – tandem mass spectrometry</td>
</tr>
</tbody>
</table>
LDH…………Lactate dehydrogenase
LDL…………Low-density lipoprotein
LFQ…………Label-free quantification
MI…………Myocardial infarction
MIF…………Macrophage migration inhibitory factor
MSC(s)………Mesenchymal stem/stromal cell(s)
MV(s)………Microvesicle(s)
MVB…………Multivesicular body
NAC…………N-acetylcysteine
NO…………Nitric oxide
NTA…………Nanoparticle tracking analysis
PFP…………Platelet-free plasma
PSM…………Peptide-spectrum match
RIC…………Remote ischaemic conditioning
RISC…………RNA-induced silencing complex
RISK pathway………Reperfusion Injury Salvage Kinase pathway
ROS…………Reactive oxygen species
RS-AFSC(s)………Round-shaped amniotic fluid stem cell(s)
SAFE pathway………Survivor Activating Factor Enhancement pathway
SDF1………Stromal cell-derived factor 1
SDS…………Sodium dodecyl sulphate
SEC…………Size-exclusion chromatography
SEM…………Standard error of the mean
sEV(s)…………Small extracellular vesicle(s)
SS-AFSC(s)………Spindle-shaped amniotic fluid stem cell(s)
STEMI…………ST-elevation myocardial infarction
TEM…………Transmission electron microscopy
TRPS…………Tunable resistive pulse sensing
TTC…………Triphenyl tetrazolium chloride
UC…………Ultracentrifugation
VEGF…………Vascular endothelial growth factor
VLDL…………Very low-density lipoprotein
Chapter 1. Background

1.1. Coronary artery disease (CAD)

Cardiovascular diseases remain the highest ranked cause of death globally and coronary artery disease (CAD) itself accounts for > 15% of all deaths in the world\(^1\). The culprit of CAD is the development of atherosclerotic plaques in the coronary arteries. Atherosclerosis is an inflammatory vascular disease caused by multiple factors including and not limited to shear stress- and turbulence-induced vascular changes, endothelial dysfunction, immune cell infiltration and lipid build-up\(^2\). This leads to coronary stenosis which clinically manifests as chest pain and discomfort. It is believed that atheromatous lesions have increased prevalence due to the present-day lifestyle and diet but, intriguingly, studies in mummies from various geographical locations documented the presence of calcified plaques > 3 millennia ago\(^3\). This may indicate a form of natural predisposition to the disease in humans. In support of this, mice, unlike humans, do not spontaneously develop atherosclerosis or acute coronary events and knockout of genes combined with a defined diet are required for development of murine atherosclerosis model organisms\(^4,5\). This questions our understanding of CAD and suggests further scrutiny of the mechanisms is needed along with development of new treatments to tackle the sequelae of CAD.

1.2. Myocardial infarction

The acute manifestation of CAD is myocardial infarction (MI). MI is characterised by blockage of a coronary artery most frequently due to an atheromatous lesion rupture. Currently, the only clinically available treatment for acute MI is swift unblocking of the artery by pharmacological (administration of a thrombolytic\(^6\)) or, more frequently, by mechanical means (balloon angioplasty and stent placement\(^7\)) to reperfuse the myocardium by restoring the blood flow. Due to the advancement of medical practice, the time between MI onset and reperfusion (time-to-reperfusion) has decreased significantly since the first use of percutaneous coronary angioplasty in 1977\(^8\), reducing the
occurrence of fatal MI events\textsuperscript{9}. However, surviving an acute coronary episode results in adverse remodelling of the heart and development of heart failure which itself leads to increased morbidity and mortality\textsuperscript{10}. Heart failure development after suffering an MI is largely determined by the final myocardial infarct size\textsuperscript{11,12}. Hence, salvaging the ischaemic myocardium using a cardioprotective intervention has been a long-sought goal in the cardiovascular medicine with the aim of improving patients’ quality of life post MI.

\subsection*{1.3. Ischaemia/reperfusion injury and cardioprotection}

A series of physiological changes occur during MI which are a consequence of the deprivation of a region of the myocardium of oxygen and nutrients and the following reperfusion. These two stages are closely linked, and they lead to a heart damage commonly referred to as ischaemia-reperfusion injury (IRI).

During ischaemia, the lack of oxygen leads to a switch to anaerobic metabolism. This causes a surge in the intracellular $\text{Ca}^{2+}$ secondary to lowering of the pH and ATP depletion\textsuperscript{13,14}. In the case of sustained ischaemia (24 h) the necrosis of the myocardium is substantial with histological appearance of cell fragmentation and karyolysis\textsuperscript{15}. The length of ischaemia is an important determinant of the extent of IRI after reperfusion\textsuperscript{16}. In fact, even 15 min of ischaemia can induce pronounced metabolite changes (65\% decrease in ATP, 56\% reduction in creatine phosphate and 331\% increase in lactate in dogs\textsuperscript{17}). As depicted in Figure 1-1 there is an exponential decay in the amount of salvageable myocardium with increasing ischaemic time. Although timely reperfusion is often achieved nowadays, it is highly unlikely that an angioplasty could be performed < 20 min after the coronary occlusion which is the ischaemic duration the heart could withstand without being irreversibly injured\textsuperscript{18}. Therefore, treatments additive to reperfusion alone should be sought.
In 1972, Maroko et al. first discovered that unblocking of a previously occluded artery and reperfusing the oxygen-deprived myocardium can ameliorate myocardial injury and improve the left ventricle wall motion\textsuperscript{15}. Reperfusion of the coronaries salvages the ischaemic myocardium partially by reintroducing blood supply but, paradoxically, it also acts as an insult to the heart causing lethal reperfusion injury which could account for up to 50\% of the resulting infarct\textsuperscript{19–23}. During reperfusion there is a burst of oxygen and reactivation of the oxidative phosphorylation leading to reactive oxygen species (ROS) production\textsuperscript{24}, ion and osmotic disbalance and cell swelling\textsuperscript{14,18}. Restoration of extracellular pH leads to cytoplasm-sarcoplasmic reticulum Ca\textsuperscript{2+} oscillations driving hypercontracture, mitochondrial Ca\textsuperscript{2+} overload and eventually formation of the mitochondrial permeability transition pore (mPTP) resulting in mitochondrial dysfunction, further Ca\textsuperscript{2+} dysregulation, severe contracture and dramatic reduction in ATP synthesis capacity resulting in cell death\textsuperscript{14,25}. Definitive proof of the existence of reperfusion injury has come from experiments where interventions were applied at the onset or during early reperfusion leading to reductions in the final infarct size\textsuperscript{23,26}. Although a
sufficiently long duration of ischaemia will inevitably result in 100% cell death of the myocardium at-risk, for shorter ischaemic times the extent of reperfusion injury-induced cardiomyocyte death could in fact exceed the ischaemia-induced cell injury. This was demonstrated in vitro where reperfusion of ischaemic embryonic chick myocytes resulted in > 4 times increase in cell death compared to sustained ischaemia. This was further confirmed in vivo in rabbit hearts, similarly demonstrating that ischaemia alone cannot induce DNA fragmentation (i.e. cell death), but that this is only observed in presence of reperfusion.

Therefore, a cardioprotective therapy adjunctive to reperfusion aimed at reducing IRI could provide enormous benefits. Such a cardioprotective therapeutic could also hold great potential for reducing the economic burden on the health system as demonstrated by a recent modelling study. Unfortunately, success in the field has been mostly restricted to the numerous preclinical studies and some small-scale clinical trials. No cardioprotective intervention has reached the clinic despite the multitude of efforts in the field. Nevertheless, revisiting old hypotheses, uncovering IRI mechanisms and designing novel targeting approaches are driving the field forward and it is still believed that cardioprotection has potential to make a great impact on clinical practice.

1.4. The present and future of cardioprotection

Currently there is general agreement that the most powerful way of protecting the heart from IRI is ischaemic preconditioning (IPC). IPC, discovered by Murry et al. in 1986, involves brief, intermittent ischaemia-reperfusion (IR) cycles applied before the index ischaemic event. A number of alternatives to this approach have been developed. For instance, a more clinically relevant variation is ischaemic postconditioning (IPost) whereby the intermittent IR episodes are applied at the onset of reperfusion. The most promising alternative has been the application of remote ischaemic conditioning (RIC) which requires induction of brief IR episodes in a remote region of the heart or even in another organ. This approach appeared to have the best potential for clinical application as it could be performed non-
invasively (e.g. using a blood pressure cuff) during or even after the myocardial ischaemia. Unfortunately, in clinical trials, RIC proved neutral in the setting of bypass grafting\textsuperscript{45}, elective cardiac surgery\textsuperscript{46} and, very recently, in a large trial in patients with ST-segment-elevation MI (STEMI)\textsuperscript{47}, despite the numerous small trials indicating benefits of RIC\textsuperscript{30}.

Nevertheless, research into methods of cardiac conditioning led to important discoveries about the mechanisms of IRI and the routes for targeting it pharmacologically. Frequently, the aim of using cardioprotective agents is to target the ion disbalance during IRI\textsuperscript{48}, to directly inhibit cell death pathways\textsuperscript{49} or to activate intracellular cytoprotective signalling pathways known as the Reperfusion Injury Salvage Kinase (RISK) pathway (involving extracellular signal–regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K))\textsuperscript{50} as well as Survivor Activating Factor Enhancement (SAFE) (acting through the signal transducer and activator of transcription 3 (STAT3))\textsuperscript{51}. Numerous experiments have demonstrated that these are effective in animal models\textsuperscript{9,39,52}.

Unfortunately, these approaches have also been largely unsuccessful in clinical trials to date\textsuperscript{31–33,36}. As a consequence, a debate on the feasibility of the search for cardioprotective therapies has emerged and it is still ongoing\textsuperscript{34–37,39,40}. The main determinants of the clinical drug failures are thought to be numerous including improper design of clinical trials\textsuperscript{34,40}, presence of comorbidities in humans which is not matched by the animal models used preclinically\textsuperscript{9,53} and lack of consistent and reproducible preclinical data on robust cardioprotection\textsuperscript{9,40,54}. Nevertheless, new promising approaches are emerging. For instance, drugs that selectively target oxidative phosphorylation enzymes in the mitochondria thereby reducing ROS production and alleviating oxidative damage during reperfusion have recently shown promise in preclinical studies\textsuperscript{55–57}. Additionally, inhibiting emerging cell death pathways in IRI, such as pyroptosis and necroptosis\textsuperscript{9,52}, may also provide another route to cardioprotection.

One of the most intriguing novel paradigms in the cardioprotection field is the use of multitargeted therapies – using two or more drugs, nucleic acids or biologics which can act in synergy to reduce IRI and prevent its adverse consequences\textsuperscript{39,58}. For instance, it has been demonstrated that using a
combination of drugs targeting different cell death pathways (i.e. necroptosis and apoptosis) prior to ischaemia and during reperfusion in an in vivo model of MI in guinea pigs can decrease infarct size further than each monotherapy\(^5\). Additive effects for infarct size reduction have also been shown in rats in vivo for platelet P2Y\(_{12}\) receptor inhibitors in combination with a pyroptosis inhibitor\(^6\) or an extracellular DNA-degrading endonuclease enzyme\(^7\). Targeting multiple pathways was also effective in an in vivo model of IRI in rabbits where administration of an agonist of glucagon-like peptide-1 receptor (GLP1R) and the antiapoptotic and anti-inflammatory parstatin prior to reperfusion led to significant infarct size reductions, bigger than the one achieved by single treatments\(^8\). Intriguingly, this combinatorial approach has also been carried forward to clinic. Recently, in the NACIAM trial (N-AcetylCysteine In Acute Myocardial infarction) a combination therapy of N-acetylcysteine and glyceryl trinitrate was administered before and during early reperfusion demonstrating marked infarct size reductions in patients presenting with STEMI\(^9\). Yet, this trial did not compare the effects of individually administered medications to their combinatorial application. Hence, more clinical studies are currently awaited to systematically compare single and multiple therapies and evaluate the hypothesis for multitargeted cardioprotection.

An exciting novel avenue for targeting of multiple pathways is the use of extracellular vesicles which carry proteins, messenger RNAs (mRNAs) and microRNAs (miRNAs) and are capable of exerting both acute and long-term beneficial effects on the heart after MI, potentially via acting on multiple pathways\(^6\)–\(^8\).

**1.5. Angiogenesis for cardioprotection and long-term benefits**

It is believed that angiogenesis is the most important vascular remodelling process for heart repair post MI\(^6\). Additionally, vascular formation in the heart post ischaemia can be cardioprotective on its own. It was shown that cell death in the heart post MI in murine models increases gradually over days\(^7\) or even months\(^7\) after the ischaemic event. Therefore, revascularisation of the previously ischaemic heart can salvage a great
proportion of the cardiomyocytes that withstood the ischaemic period but are destined to die because of the reperfusion injury.

Scar formation in the heart post MI resembles typical wound healing and angiogenesis appears to be a natural response after an MI. Cardiac biopsies from patients with MI showed elevated hypoxia-inducible factor 1-alpha (HIF1α) expression in the heart early (< 24 h) after the onset of MI, followed by an increase in vascular endothelial growth factor (VEGF) expression, detected in the evolving infarctions 1-5 days post MI. Upregulation of the main angiogenesis players such as VEGF and VEGF receptors was also shown in an angiogenesis model of chronic coronary artery ligation in rats. In fact, angiogenic processes are evident in the first 1-2 weeks post MI in dogs but they decrease by 4 weeks after the ischaemic event. This indicates that angiogenesis may indeed be a cardioprotective physiological response of the organism occurring early on after MI to respond to the IRI events. It is therefore believed that a therapy to increase these angiogenic processes and re-establish the blood flow to the damaged region of the heart post MI may be beneficial.

In addition to the cardioprotective effect, formation of new blood vessels in the heart post MI will ensure access of inflammatory cells to clear the debris in the damaged portion of the heart and to initiate formation of a stable scar. In fact, there is a complex interplay between angiogenesis and inflammatory response which are dependent on one another. This is evidenced by experiments in pigs demonstrating that capillary formation within the necrotic areas of the heart after microembolisation is dependent on the presence of an active immune response as immunosuppression markedly reduced angiogenesis.

Interestingly, angiogenesis may also be important for counteracting pathophysiological cardiac hypertrophy and achieving adaptive cardiac overgrowth. Studies showed a fine balance exists between cardiac hypertrophy and angiogenesis. Using a cardiac overgrowth model of mice with inducible AKT1-overexpression, Shiojima et al. demonstrated that hypertrophy of the heart was dependent on angiogenesis and was matched by new blood vessel formation. Intriguingly, the authors showed that an intact angiogenic processes maintained adaptive cardiac growth while the use of a decoy VEGF
receptor caused a mismatch between angiogenesis and cardiac growth and development of heart failure\textsuperscript{80}. Shiojima et al. argued that angiogenesis is essential for physiological growth and when impaired may lead to fibrotic changes and cardiac dysfunction\textsuperscript{80}. Hence, a stimulation of angiogenesis post MI may be able to reduce the incidence of heart failure by shifting the heart remodelling to an adaptive cardiac growth.

Therapeutic proangiogenic interventions have been investigated in animal models of MI. For instance, dogs subject to chronic MI by laser ablation-induced stenosis and artificial thrombus insertion had reduced infarct size and improved left ventricular ejection fraction after intracoronary administration of basic fibroblast growth factor (FGF2)\textsuperscript{81}. This was attributed to an increased number of both capillaries and arterioles in the heart\textsuperscript{81}. Perivascular injection of hydrogel-encapsulated FGF2\textsuperscript{82} or osmotic pump-delivered VEGF\textsuperscript{83} in pigs with chronic MI induced by circumflex artery constriction also confirmed a potential benefit of proangiogenic therapies for IRI: coronary flow and fractional shortening were improved, while left ventricular end-diastolic pressure was reduced. Currently, there is further interest in using miRNAs\textsuperscript{84}, gene\textsuperscript{85} and stem cell\textsuperscript{86} proangiogenic therapies. One of the most promising novel ways of promoting post-ischaemic angiogenesis is through use of extracellular vesicles as proangiogenic nanoparticles due to the diverse cargo they contain and the potential multitargeted beneficial effects in the setting of MI\textsuperscript{87–90} (see also sections 1.4. and 1.6.3.).

1.6. Extracellular vesicles

“Extracellular vesicles” (EVs) is a generic term encompassing various populations of lipid-bilayer vesicles released into the extracellular space by all cell types\textsuperscript{91}. There is still an ongoing discussion about the nomenclature of EVs but two main subgroups according to their size and origin are recognised to exist: 1) microvesicles (MVs) (100-1000 nm) and 2) exosomes (30-150 nm)\textsuperscript{92,93}. MVs are secreted by budding of the plasma membrane while exosomes have endocytic origin being generated in an intracellular organelle called multivesicular body (MVB) (Figure 1-2A)\textsuperscript{89,94–96}. A third group labelled “apoptotic bodies” is sometimes defined as a separate sub-classification of
EVs which includes large vesicular fragments formed by plasma membranes during an apoptotic process\textsuperscript{97}.

Very recently, a novel group of nanoparticles released by cells in the extracellular space has been identified, namely “exomeres”\textsuperscript{98}. These are smaller than exosomes and MVs (~35 nm in diameter) and possess different protein composition to the aforementioned EVs\textsuperscript{98}. It should be noted, however, that exomeres are not considered an EV group since they are not membrane-encapsulated\textsuperscript{98}.

EVs have received great attention in recent years and interest in their biological functions is growing rapidly in many fields such as cancer biology\textsuperscript{92,99}, stem cell biology and regeneration\textsuperscript{100,101}, liver biology\textsuperscript{102}, infectious disease\textsuperscript{103}, metabolic disease\textsuperscript{104} and cardiovascular biology\textsuperscript{9,87,89,90,105}. EVs can be secreted by virtually every cell in the body and they may serve as paracrine, endocrine or autocrine signals\textsuperscript{106}. They can be isolated from biological fluids such as plasma, serum, breast milk, cerebrospinal fluid, semen, urine, and also from conditioned cell culture medium\textsuperscript{107–114}.

Protocols for EV isolation for experimental studies usually rely on the physical and biochemical properties of the vesicles and not on their origin (e.g. endosomal versus plasma membrane). Therefore, in line with multiple recent reports\textsuperscript{115–117} and guidelines\textsuperscript{118}, in the current thesis the term \textit{small extracellular vesicles} (sEVs) was adopted to label vesicles isolated from large vesicle-devoid blood plasma or conditioned cell culture medium (i.e. a population of EVs comprising predominantly of exosomes as identified by the characterisation methods).

sEVs are characterised by the presence of CD9, CD63 and CD81 tetraspanin membrane markers on their surface\textsuperscript{116}. Recent reports indicate that other proteins, such as syntenin-1, may be even more specific to the MVB-generated vesicles\textsuperscript{115,116} and the use of multiple markers to confirm the presence of sEVs is generally advised\textsuperscript{118}. sEVs have been shown to carry various proteins, mRNAs and miRNAs but there is still a debate in the field about whether they contain DNA (Figure 1-2B)\textsuperscript{119–123}. Despite the earlier indication that sEVs carry single-stranded and double-stranded DNAs originating from the nucleus or even the mitochondria\textsuperscript{120,121,123}, a fascinating
recent report isolated highly-pure sEVs using high-resolution density gradient purification and demonstrated that sEVs are likely DNA-free\textsuperscript{115}.

It is believed that considerable heterogeneity in the isolated sEV populations exists due to the mostly physical criteria used for isolation. Vesicles originating from a single cell source could consist of various subpopulations with different protein and RNA cargo but there is still limited evidence for this, mainly due to difficulties in separating EV subtypes of similar sizes or densities\textsuperscript{116,124}.

\textbf{Figure 1-2. Endosomal sEV secretion and morphology}

A – Simplified schematic of generation and secretion of exosomes. 1 – vesicles form by intraluminal invagination of the MVB membrane. 2 – formed vesicles reside in the MVB. 3 – MVB translocates and fuses with the cell membrane, thus releasing the internal luminal vesicles in the extracellular space, \textit{i.e.} an active cellular release of exosomes. B – Schematic of an sEV. sEVs are phospholipid bilayer vesicles and contain various nucleic acids as well as intraluminal and membrane-anchored proteins (depicted).
1.6.1. Exosomes as major components of the sEV population: formation and release

sEVs are arguably the most studied type of EVs. Most methods used to isolate sEVs (including those that were used here) harvest predominantly exosomes from biological fluids or conditioned medium. In 1981 Trams et al. reported the existence of membrane vesicles containing ATPase and 5’-nucleotidase released from murine and human neoplastic cells in culture\textsuperscript{125}. They proposed that these EVs are labelled exosomes\textsuperscript{125}. Soon afterwards, two reports confirmed the release of endosomal vesicles from sheep\textsuperscript{94} and rat\textsuperscript{95} reticulocytes. In an elegant biochemical and electron microscopy study, Harding et al. demonstrated that gold-labelled transferrin binds to the surface transferrin receptors which are firstly internalised and localise to multivesicular endosomes (i.e. MVBs) and later released in vesicles in the extracellular space\textsuperscript{95} (Figure 1-2A,B). Subsequently, many players in MVB vesicle formation and cargo sorting and loading have been identified with the endosomal sorting complex required for transport (ESCRT) proteins being central for exosome biogenesis\textsuperscript{117,126–128}. Other ESCRT-independent components are also necessary for exosome formation including neutral sphingomyelinases\textsuperscript{129}, while Rab GTPases were identified as the most important players involved in MVB trafficking and exosome release in the extracellular space\textsuperscript{130–132}.

Understanding exosome biogenesis is a long-sought goal in the field. Identification of specific players in exosome formation and release will allow for development of tools to inhibit or activate these targets which may provide definitive answers as to whether exosomes are the active mediators of physiological or pathological processes. Until now multiple reports have used inhibitors of exosome secretion (e.g. neutral sphingomyelinase inhibitors or mutant/silenced Rab GTPases) or activators of exosome release (e.g. drugs that interfere with ion transport and signalling) but these were found to be non-specific or to interfere with secretion of larger EVs as well\textsuperscript{117,118}. Therefore, the search for inhibitor/activator of exosome release continuous and the outcome will be crucial for future studies in the field.
1.6.2. sEV isolation and purity

A major debate in the EV field concerns the purity of the obtained vesicle samples due to the inherent limitations of the isolation methods used\textsuperscript{109,118}. Usually, techniques for the isolation of vesicles from biological fluids or conditioned medium provide an sEV-enriched sample with various degrees of contaminating factors present. The most common methods for isolation of sEVs rely on separation of vesicles based on their density (e.g., ultracentrifugation, density gradient or sucrose cushion centrifugation), their size (e.g., size-exclusion chromatography), expression of sEV-specific markers (e.g., immunoaffinity capture) or a simple volume exclusion involving a relative concentration of vesicles in the isolates (e.g., precipitation using polymers such as polyethylene glycol) (see Table 1-1 for an overview of the most common sEV isolation methods and further discussion below). Typical contaminants in the samples comprise of non-EV proteins\textsuperscript{133,134}, RNAs\textsuperscript{135–137} and lipoproteins\textsuperscript{134,138–142}. These are co-purified with exosomes by virtually all methods used currently\textsuperscript{133,141,143,144} and could confound the outcomes of functional studies that are conducted subsequently. Co-purification occurs due to similarities in size or density of the sEVs and the other constituents of the samples of origin (Table 1-1, Figure 1-3). It has also been suggested that contaminants and sEVs might physically interact\textsuperscript{139,140,145}. 
Figure 1-3. Size-density plot of sEVs and common sEV contaminants

The size (x-axis) or density (y-axis) of each lipoprotein type, sEVs and protein aggregates is shown by the span of the bubbles on the graph. Information collected and adapted from Simonsen et al.\textsuperscript{139}. HDL – high-density lipoproteins. LDL – low-density lipoproteins. IDL – intermediate-density lipoproteins. VLDL – very low-density lipoproteins. sEVs – small extracellular vesicles.
Table 1-1. Overview of some of the most commonly used methods for sEV isolation

Information adapted from Coumans et al. (2017)^146.

<table>
<thead>
<tr>
<th>Method</th>
<th>Principle</th>
<th>Expected contaminants</th>
<th>Observed contaminants</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultracentrifugation</td>
<td>Mass-density, shape and size relative to the density of the medium</td>
<td>Some lipoproteins and protein aggregates</td>
<td>Some soluble proteins Lipoproteins</td>
<td>Inexpensive procedure (except the equipment) Relatively straightforward and rapid</td>
<td>Expensive equipment Particle aggregation and disruption Variations in the level of contamination due to the operator and the nature of the sample</td>
</tr>
<tr>
<td>Density gradient centrifugation</td>
<td>Density</td>
<td>High-density lipoproteins</td>
<td>Some lipoproteins</td>
<td>Provides sEVs with better purity</td>
<td>Laborious and time-consuming Particle aggregation and disruption Yield is likely to be lower</td>
</tr>
<tr>
<td>Size-exclusion chromatography</td>
<td>Size</td>
<td>Large lipoproteins or protein aggregates</td>
<td>Lipoproteins</td>
<td>Rapid isolation Intact and relatively pure vesicles (in serum-free conditions) Commercially available columns ensure reproducibility Relatively inexpensive</td>
<td>Processing of the sample prior to isolation may be time-consuming (e.g. with large volumes of culture medium) The presence of serum in conditioned medium can lead to severe contamination of the sEVs Obtained sEVs are usually dilute</td>
</tr>
<tr>
<td>Immunoaffinity capture</td>
<td>Specific antigen</td>
<td>Only species that directly associate with sEVs</td>
<td>Soluble proteins</td>
<td>Provides specific subpopulation of vesicles</td>
<td>Yield is likely to be low Can be time-consuming</td>
</tr>
<tr>
<td>Precipitation</td>
<td>Volume exclusion (i.e. concentration)</td>
<td>Proteins and lipoproteins</td>
<td>Soluble proteins Lipoproteins</td>
<td>Inexpensive Quick and simple to perform</td>
<td>Contaminants may be present in excess</td>
</tr>
</tbody>
</table>
Lipoproteins, as major potential contaminants in the sEV isolates, are mainly thought to cause issues when using blood plasma or serum as a starting sample. However, analysis of the main EV isolation techniques suggested that almost half of sEV isolations from conditioned cell culture medium are performed in serum-supplemented conditions\textsuperscript{109}. Since lipoproteins (mainly HDL and LDL\textsuperscript{139}) are suggested to account for an astronomical $\sim 10^{16}$ particles per 1 ml of plasma, 6-7 orders of magnitude above the sEV amounts\textsuperscript{139}, and the starting volumes of conditioned medium could sometimes exceed 100 ml\textsuperscript{109}, a significant lipoprotein contamination in downstream experiments with the isolated vesicles could be expected. Given the high number of lipoproteins in blood, it is hardly surprising that they were also shown to physically interact with EVs making the two virtually inseparable by density or size\textsuperscript{140}.

Soluble non-sEV proteins can also contaminate sEV isolates which is most obvious when using non-specific methods for sEV harvesting such as polymer precipitation. This has been shown by the presence of non-sEV plasma proteins in the harvested sEV samples such as albumin, complement factors and fibrinogen\textsuperscript{145,147}. One study even failed to detect any sEV markers in a precipitation isolate despite identifying abundant plasma proteins\textsuperscript{147}. Non-sEV proteins may also be found in sEV isolations from conditioned cell culture medium\textsuperscript{148}. A comparative study of different methods for isolation of sEVs from low-serum-supplemented conditioned medium found that precipitation sEV isolates contain considerably lower levels of sEV markers compared to ultracentrifugation isolates despite a 3-8 times higher total protein yield\textsuperscript{148}. Strikingly, the authors reported that precipitation methods yielded samples with sEV markers that were even less enriched than a total cell lysate\textsuperscript{148}. This is indicative of the presence of an excess of non-sEV proteins. In another study\textsuperscript{149}, a sample of isolated serum EVs reportedly contained almost 30\% of the total serum protein content\textsuperscript{150,151}, a quantity that clearly indicates marked protein contamination. In support of this we have shown that the isolated sEVs should theoretically contain $\sim 0.001\%$ of the total serum protein (or $> 4$ orders of magnitude lower than the one reported in the aforementioned study)\textsuperscript{89}.

Even when using serum-free conditioned medium, some non-EV proteins may be co-purified with sEVs. It is usually easier to identify contaminating
soluble proteins in serum-supplemented conditions, where the species of origin of the contaminant is frequently different from the cultured cell \textit{(e.g. bovine serum versus human or murine cultured cell)}. As explained above, contaminants in these conditions can also be identified due to the fact that they are very unlikely to be produced by the cultured cells \textit{(e.g. lipoproteins)}. However, this is not straightforward when analysing serum-free sEV isolates. In that case, a score known as particle-to-protein ratio of an sEV sample can be used as indirect evidence for the presence of soluble proteins$^{89,133}$. Harvests with ratios of $\sim1\times10^{10} - 3\times10^{10}$ particles per 1 µg of protein have been suggested to be pure sEV isolates$^{89,133}$. However, particle/protein ratios reported in the literature for serum-free conditioned medium isolations rarely meet these criteria, regardless of the isolation method used$^{152–154}$. Notably, this ratio should be interpreted with caution due to operator-dependent variations$^{155}$, variable particle enumeration methods and diverse selections of settings in each report$^{133,155–157}$, as well as the presence of other particulate material such as lipoproteins$^{140}$ (see also discussion in section 3.5. and 4.5.).

sEVs contain miRNAs that are thought to mediate a variety of their functions by regulation of protein expression in a target cell$^{119}$. However, some (or most) of the miRNA in an sEV sample can also be non-vesicular. Arroyo \textit{et al.} in an elegant study employed size-exclusion chromatography to fractionate blood plasma or serum and demonstrated that about 2/3 of the plasma/serum miRNAs are non-vesicular, \textit{i.e.} eluting with particles of $< 5$ nm diameters potentially representing miRNAs associated with argonaute-2 (AGO2) protein$^{135}$. Only $\sim15\%$ of the miRNA was found eluting with particles of $\sim100$ nm diameters, putatively vesicles$^{135}$. In addition to that, techniques such as precipitation were shown to result in isolates enriched in AGO2$^{148}$ while density-based sEV isolation can lead to co-purification of HDL which was shown to bind to non-sEV miRNAs$^{141}$. Hence, the purification technique may influence majorly downstream experiments with sEV-associated miRNAs. Strikingly, stoichiometric analysis of the miRNA content of sEVs in plasma, seminal fluid and conditioned medium suggested that even the most abundant miRNAs are present at 1:9 miRNA:sEV ratio while this could reach 1:50,000 for a less abundant miRNA$^{108}$. The precise ratios are arguable, however, due to the contaminating lipoprotein particles$^{140}$ which could lead to
underestimation of the number of miRNA per sEV in plasma. Nevertheless, a recent high-resolution density separation of different EV subtypes identified a lack of various miRNA associated proteins such as AGO, Drosha and Dicer in highly-purified sEV samples\(^{115}\). Additionally, many abundant extracellular miRNAs were found to be depleted from highly-pure, density-gradient-isolated sEVs\(^{115}\). Therefore, as argued by the authors, the presence of an miRNA in the sEV isolate is insufficient to confirm whether it is specifically incorporated in sEVs or co-isolated as a contaminant.

Hence, characterisation of the obtained sEV samples and their purity is required and recommendations for this have been released\(^{110,118,146}\). Typical characterisation may include particle enumeration and size measurements\(^{156}\), electron microscopy imaging\(^{94,95,158}\), protein quantification and determination of specific sEV marker content (e.g. CD9, CD63, CD81 tetraspanins)\(^{159}\), together with confirmation of the absence of non-sEV proteins (e.g. APOB (apolipoprotein B), associated with lipoproteins; HSP90B1 (endoplasmin, gp96), associated with endoplasmic reticulum origin; ACTN4 (alpha-actinin-4), associated with large- and medium-sized EVs)\(^{118}\).

Currently, there is no ideal method for isolation of completely pure sEVs with high yields in a simple procedure. Usually, investigators chose either increasing the yield at the expense of compromised purity, or vice versa. Among the methods used for vesicle isolation, size-exclusion chromatography (SEC) is deemed to be superior to most other methods as it does not require high-speed centrifugation, polymer-based precipitation, lengthy procedures or expensive equipment, and it preserves vesicular structure well\(^{145,157,159–161}\). It performs well in purifying sEVs from soluble protein and some lipoproteins (e.g. HDL) but may not be optimal when there is a large amount of APOB\(^+\) lipoproteins such as LDL, VLDL and chylomicrons\(^{134,140}\) (see also chapter 3). Nevertheless, when using appropriate starting conditions devoid of the aforementioned lipoproteins, SEC holds enormous potential for the isolation of high yields of sEVs with superior purity to that of vesicles harvested by other methods. This method is also versatile as it provides the operator with a choice of different eluent fractions with varying sEV yield-to-purity ratios, thus allowing one to either maximise the sEV yield or minimise the contaminating factors, as preferred (see section 2.2. for details of this methodology).
Overall, the purity of the isolated sEVs should be very carefully examined in experimental settings and any functional effects observed subsequently should be interpreted while taking this into consideration. Due to the multitude of the sEV isolation approaches and sometimes their complexity, protocol and operator-dependent variations arise. Therefore, one of the main goals of the EV community is standardisation of techniques, clear and transparent reporting of the methodology and systematic and rigorous experimentation. One of the major initiatives established in relation to these issues is the EV-TRACK consortium: an online database aimed at improving experimental reproducibility, encouraging rigorous experimentation and robust data acquisition and, most importantly, advocating transparency in reporting. The latter is considered to be one of the most important parameters to drive the field forward. EV-TRACK database provides an “EV-METRIC” score for each EV experiment that is submitted to the database. EV-METRIC reflects the amount of reported information about the protocols and the characteristics of the obtained EVs. Submissions to EV-TRACK may include published material, data submitted for publication or even pilot experiments to guide the authors and provide them with a quality metric of their procedures and reporting. EV-TRACK is also a particularly useful reference database for expected yields and purities for a variety of EV isolation protocols, and it can also be considered a training platform. In this report, EV-METRIC was used as an indication of the quality and robustness of the sEV isolation, characterisation and functional studies.

1.6.3. sEVs and cardioprotection

Generally, EVs have been explored in two broad research lines in the cardiovascular field: as biomarkers for cardiovascular diseases and as functional carriers of proteins and RNAs altering a recipient cell physiology. In the MI setting, any alterations in the plasma EV number, their protein cargo or miRNA content could be exploited as an injury biomarker with the advantage of having blood as an easily accessible and ample source of EVs. With regard to the function of sEVs in the cardiovascular system, the list of published articles is lengthy with a multitude of roles in cardiovascular
physiology and pathophysiology reported in the literature\textsuperscript{87,89,90,104,106,167}. With their diverse protein and RNA cargo\textsuperscript{115,116,118,122}, sEVs represent an exciting potential multitargeting therapeutic for cardioprotection, proangiogenesis and heart regeneration and repair\textsuperscript{9,39,87–90,168,169}. Due to the increased interest in exploiting sEVs as biomarkers or therapeutics, guidelines for sEV research in cardiovascular science and medicine have been published\textsuperscript{88,146} in addition to the general International Society for Extracellular Vesicles methodological directions\textsuperscript{118}.

EVs have been studied as potential mediators of IPC and RIC. The rationale behind these studies was the presence of cardioprotective miRNAs\textsuperscript{170,171} or proteins\textsuperscript{172,173} in the plasma EVs and also the observations that RIC increases the number of plasma sEVs\textsuperscript{64,171}. Our laboratory was the first to suggest that EVs might mediate RIC\textsuperscript{174}, while Giricz \textit{et al.} were the first to provide evidence for a role of EVs in IPC, showing that coronary perfusate of isolated rat hearts collected during an IPC can reduce infarct size if perfused in a naïve recipient heart before IRI\textsuperscript{175}. This cardioprotective effect was lost when the coronary perfusate was depleted of EVs\textsuperscript{175}. It should be noted, however, that authors did not perfuse the recipient hearts with isolated EVs alone. Thus, a protective effect of other factors cannot be excluded since depletion of sEVs involved dialysis, filtrations and centrifugations which may have also led to depletion of other, non-EV cardioprotective factors released during IPC. Recently, a role for EVs as mediators of RIC in kidney IRI was proposed but no mechanism was identified and thorough characterisation of EVs was not presented to provide definitive conclusions\textsuperscript{176}.

Overall, evidence for a role of sEVs in heart conditioning is sparse. In fact, systematic \textit{in vitro}, \textit{ex vivo} and \textit{in vivo} studies from our group showed that the sEVs derived from rat blood plasma have cardioprotective potential regardless of whether a prior RIC was applied to the donor rat or not\textsuperscript{64}. In addition, it has been increasingly recognised that blood-derived sEVs are markedly contaminated with various lipoproteins and soluble protein and their nature is likely heterogeneous comprising sEVs originating from numerous cell types\textsuperscript{133,140,141,144}. Therefore, their use as a therapeutic for IRI is unlikely to achieve success due to these limitations (see also blood plasma sEV isolation analysis in chapter 3).
There are various single cell sources of sEVs which may have beneficial effects in IRI setting with stem/progenitor cell sEVs attracting by far the most attention. Interest in the potential of sEVs for cardioprotection and heart repair and regeneration stemmed from studies using mesenchymal stem/stromal cells (MSCs). MSCs are adherent, multipotent foetal or adult cells capable of differentiating into cells of the cardiovascular system amongst many others (Figure 1-4)\textsuperscript{177}. Isolated for the first time about 50 years ago\textsuperscript{178}, MSCs have been well-documented to exhibit beneficial functions on the heart and to aid cardiac repair\textsuperscript{179–183}. Nevertheless, cells injected into the heart were shown to be poorly retained and engrafted, regardless of the route of their administration or the cell type used\textsuperscript{184–187}, indicating that the benefits of MSCs may not be entirely due to differentiation into new cardiomyocytes or vascular cells as originally thought (Figure 1-4). In line with this, in the MI setting MSCs can deliver functional improvements less than 3 days after injection\textsuperscript{188} and MSC-conditioned medium alone was shown to reduce IRI-induced cell death \textit{in vitro}\textsuperscript{188} and \textit{in vivo}\textsuperscript{189}. It is now generally accepted that MSC benefits in an MI setting are largely conferred by their secretome, \textit{i.e.} via a paracrine action of the MSCs (Figure 1-4).

The first evidence for a role of MSC-derived sEV in cardioprotection was documented in 2010 when Lai \textit{et al.} demonstrated that the CD9-rich fraction of embryonic stem cell-derived MSC-conditioned medium reduced infarct size in a mouse model of IRI when administered intravenously 5 min prior to reperfusion\textsuperscript{65}. Lai \textit{et al.} were the first to show that the acute cardioprotective effects of MSC conditioned medium in IRI were entirely recapitulated by the sEV-rich fraction of the MSC conditioned medium\textsuperscript{65}. It was later suggested by the same group that the mechanism behind these effects is through a multifaceted roles of sEVs including AKT and GSK3 phosphorylation, reduction in proapoptotic phosphorylated c-JNK and reduction in infiltrating neutrophils in the heart\textsuperscript{190}. Observations in these studies that sEVs are protective in a model of IRI in isolated perfused hearts indicates that they are acting directly on the cells of the heart and the effects are likely independent of the immune system, neural pathways or endocrine responses\textsuperscript{65,190}.
Figure 1-4. Mechanisms of the cardioprotective and regenerative benefits conferred by mesenchymal stem/stromal cells (MSCs)

MSCs are multipotent progenitors that can differentiate into numerous cell types including cardiomyocytes and vascular cells (endothelial cells and vascular smooth muscle cells). Despite the initial belief that this differentiation can be exploited to drive heart repair and regeneration, there is a general agreement among scientists working with MSCs that their benefits for cardioprotection, angiogenesis and heart regeneration are mainly conferred by the paracrine factors MSCs secrete, including extracellular vesicles and various soluble proteins.

In an attempt to improve the potency of the harvested MSC sEVs, various engineering approaches have been employed. For instance, sEVs were found to be more efficient in improving heart function when intravenously injected in rats with permanent MI, if they are sourced from AKT-overexpressing human umbilical cord MSCs as opposed to naïve MSCs. AKT-overexpressing MSC-derived sEVs were also more potent in inducing migration and proliferation of endothelial cells as well as in promoting vascular formation in chick allantoic membrane assay.

Similarly, in another study Yu et al. overexpressed GATA-4 in rat bone marrow MSCs with the premise that these cells will resemble cardiac phenotypes better and may have improved secretome with regard to cardioprotection and cardiac repair. They indeed showed that sEVs produced by these MSCs are more effective in reducing cell death of neonatal...
cardiomyocytes undergoing hypoxia, but also in decreasing apoptosis and fibrosis and improving ejection fraction and fractional shortening in rat hearts subjected to permanent MI\textsuperscript{192}.

Interestingly, the use of stem/progenitor cells which more closely resemble myocardial cells for cardioprotection studies has gradually been accepted as one of the most promising approaches in this field. Despite the reports for cardioprotective effects of sEVs derived from other promising stem cell populations such as CD34\textsuperscript{+} peripheral blood stem cells\textsuperscript{193} or embryonic stem cells\textsuperscript{194}, sEVs from resident cardiac progenitor cells\textsuperscript{66,195,196} are considered to hold better potential for cardioprotection due to the developmental origin and cardiac nature of these cells\textsuperscript{197,198}. For instance, such cells are cardiac progenitor cells (CPCs), harvested from human atrial appendages\textsuperscript{196}, and cardiosphere-derived cells (CDCs), isolated from human heart biopsies\textsuperscript{66} or animal heart explants\textsuperscript{199}, which have been studied extensively recently\textsuperscript{66–68,152,196,199–203}. Direct comparisons between CDCs and bone marrow MSCs showed a better antiapoptotic effect of CDCs in a mouse \textit{in vivo} MI model\textsuperscript{198}. CDCs were further demonstrated to release higher levels of cardioprotective and proangiogenic factors than bone marrow MSCs\textsuperscript{198}. Furthermore, another study argued that CPC-derived sEVs are more potent than bone marrow MSC-derived sEVs because of the bigger infarct size reduction and better heart function they deliver in a permanent MI model in rats \textit{in vivo}\textsuperscript{202}.

A series of successful preclinical studies\textsuperscript{66,68,199–201} and a pilot clinical trial (CADUCEUS\textsuperscript{201}) were reported for CDCs in the MI setting. Reports demonstrated improvements of heart function and reduction of infarct size upon administration of CDCs after permanent\textsuperscript{66,204} or acute\textsuperscript{199,205} MI in animals. Similarly to the MSCs, CDC secretome is thought to be the main mediator of these effects\textsuperscript{200} where EVs were reported as major effectors present in the paracrine milieu\textsuperscript{67,68,152,206}.

The first evidence for a role of vesicles in the CDC-conferred effects was published by Ibrahim \textit{et al.} who demonstrated that CDC sEVs promote endothelial cell tube formation and neonatal rat cardiomyocyte proliferation while reducing apoptosis of stressed cardiomyocytes\textsuperscript{206}. \textit{In vivo}, immunodeficient mice with permanent infarcts had improved ejection fraction
and decreased scar size when administered with CDC sEVs, an effect abrogated by pre-incubation of CDCs with the sEV-depleting drug GW4869 (neutral sphingomyelinase inhibitor). The authors argued that the mechanism involved vesicular miRNA-146a, since miRNA-146a shRNA inhibition in CDCs led to secretion of sEVs with reduced protective effects. It should be noted, that in this first study sEVs were poorly characterised and in fact ~70% of the particles in the isolates were found to be < 35 nm in diameter, which raises doubt about their sEV identity.

In fact, the use of suboptimal sEV isolation techniques and poor characterisation of the sEVs dominate published studies with CDCs. For instance, CDC-derived sEVs were shown to deliver cardioprotection by inducing phenotypic alterations in target cells such as fibroblasts and macrophages. Tseliou et al. demonstrated that dermal fibroblast derived sEVs injected 4 weeks post permanent MI were protective only when the fibroblasts were pretreated with CDC-derived sEVs. Moreover, the same group later demonstrated that treatment with CDC sEVs after reperfusion in rat and pig acute MI models reduces infarct size via selective transfer of miRNA-181b and downregulation of PKCδ in target macrophages. Although providing more information on the characteristics of the isolated vesicles, some of the reported data is conflicting. Authors demonstrated that precipitated sEVs are negative for CD63 marker, have very low CD9 marker and measured around 175 nm in diameter. Vesicles with these characteristics should theoretically represent a minority of the isolated population according to other authors following similar protocols to pre-clear the samples from large vesicles and procedures are certainly not adhering to the published guidelines. Furthermore, one of the studies found no CD81 expression in isolated CDC sEVs while CD63 expression levels were enriched in the conditioned medium compared to the isolated vesicles, which further indicates that contaminants may be present in excess. The authors did not provide explanations for these discrepancies and the identity of any impurities or heterogenous vesicular populations that might have been present remains elusive.

An additional pivotal issue for the majority of the CDC sEV studies is the timing of CDC incubation in culture. CDCs were grown for exceptionally long
periods (15 days\textsuperscript{67,206}) in serum-free conditions which induces a striking > 40% cell death\textsuperscript{206}. There is a possibility that this amount of cell death may have led to excessive passive release of intracellular components or cell membrane fragments (\textit{e.g.} apoptotic bodies). Whether this was the case remains largely unknown as authors did not investigate expression of markers typical for large apoptotic bodies (\textit{e.g.} HSP90B1\textsuperscript{96}).

Approaches to engineer improved CDC sEVs have also been studied recently. For instance, investigators modified EVs to target different organs or cells inserting a streptavidin-dioleoylphosphatidyl-ethanolamine N-hydroxysuccinimide (DOPE) conjugate (phospholipid embedding moiety) into EV membranes to serve as a bait for biotin-conjugated targeting peptides or fluorescence molecules\textsuperscript{207}. It should be noted, however, that given the impure isolation method of choice (\textit{i.e.} ultrafiltration only\textsuperscript{207}) it is possible that the phospholipid embedding moiety may also bind to contaminating proteins in the samples, similar to what we have shown occurs with lipid-conjugated membrane dyes, thus masking the true sEV incorporation\textsuperscript{138} (see chapter 8).

In view of the above, most of the CDC sEV studies suffer from a number of limitations, despite the promising data obtained with them. Further to that, there is a growing debate in the cardiovascular field regarding the existence and nature of cardiac progenitor/stem cells\textsuperscript{208}. A large number of peer-reviewed high-profile scientific articles about cardiac stem cells have been retracted or are called for retraction (\textit{e.g.} \textsuperscript{209,210} and clinical trials\textsuperscript{211}, see \textsuperscript{208} for critical analysis), while different groups used genetic lineage tracing to definitively show that endogenous cardiac stem cells are unlikely to be present in the heart\textsuperscript{212,213}.

Regardless of whether cardiac cell subpopulations can be categorised as progenitors, they are most frequently isolated from human biopsies from patients undergoing invasive cardiac procedures\textsuperscript{66,196,214}, \textit{i.e.} usually people with cardiovascular disease. This may present with a plethora of problems including the inferior potential of cells harvested from patients with some cardiovascular pathologies\textsuperscript{215} or the phenotypical and functional heterogeneity of cells obtained from different patients with CAD\textsuperscript{215}. It is currently unknown whether this may generate long-term issues related to the origin of these cells or cell-derived EVs from diseased patients.
In fact, the search for an optimal stem cell source for cardioprotective sEVs is still ongoing and there is no definitive answer as to which progenitor cell provides best results. A recent extensive report on induced pluripotent stem cells (iPSCs) indicated that younger, pluripotent (unlike the multipotent MSCs) progenitor cell populations may release sEVs with cytoprotective, proangiogenic, promigratory and antihypertrophic activity which exceeds the one provided by the parent iPSCs. However, this study did not compare the iPSC-derived sEVs with MSC-derived sEVs and therefore it remains unknown if younger iPSC sEVs are more potent than other cell sources. In fact, the dose of iPSC sEVs used by the authors to achieve the aforementioned effects was very high on all instances and the amount of vesicles administered \textit{in vivo} (~4 mg/kg on average) was ~1,000 times higher than the one required to achieve cardioprotection with a population of young embryonic stem cell-derived MSC sEVs (4 µg/kg)\textsuperscript{190}. Therefore, the superior potency of the iPSC-derived sEVs remains questionable.

Rigorous systematic comparison between the sEVs derived from different stem cells is missing and there are various ethical concerns (\textit{e.g.} using embryonic stem cells to derive MSCs\textsuperscript{65,190}) or procedural difficulties (\textit{e.g.} differentiation of embryonic stem cells\textsuperscript{65,190}, generation of iPSCs or iPSC-derived cells\textsuperscript{101,216}, cardiac biopsies from diseased patients\textsuperscript{67,68,196}) for obtaining progenitor cells. The hypothesis of using younger progenitor cell populations and the sEVs secreted by these in the MI setting has been one of the most attractive ones recently. Novel foetal sources have been explored to provide cells which are easily harvested, with optimal expansion potential and reduced ethical concerns as well as possessing better functional efficacy\textsuperscript{217,218,227,228,219–226}.

### 1.7. Amniotic fluid stem cells

Reports indicate that MSCs isolated from foetal tissues, unlike their adult counterparts, possess some degree of pluripotency, have greater expansion capacity, ~60% longer telomeres and > 5 times higher expression of telomerase\textsuperscript{225–227}. Interestingly, foetal MSCs have similarities with embryonic stem cell-derived MSCs in displaying greater proliferation potential and
telomerase activity than adult MSCs\textsuperscript{217}. In addition to the proliferative capabilities of the foetal MSCs, they have also been shown to be more potent than adult MSCs in the setting of cardioprotection, heart repair and regeneration\textsuperscript{224}. As an example for this, medium conditioned by foetal MSCs collected from the endometrium was found to have improved antiapoptotic effects than adult bone marrow MSC-conditioned medium in a model of neonatal cardiomyocyte hypoxia-induced cell death and also more pronounced endothelial cell tube formation activities \textit{in vitro}\textsuperscript{228}.

Foetal tissue MSCs can be obtained from liver, kidney, limbs etc.\textsuperscript{217}. A very attractive source of MSCs is the amniotic fluid which is rich in progenitor cells: it contains an MSC population referred to as amniotic fluid stem cells (AFSCs) whose translational potential has been well-recognised\textsuperscript{218–225}. AFSCs were shown to possess some degree of pluripotency as demonstrated by expression of markers such as OCT4 and SSEA-3/4, which suggests that they have an increased differentiation potential compared to adult MSCs\textsuperscript{225–227}. Due to their young age, AFSCs are also expected to have fewer age-related genetic mutations that may be associated with adult MSCs\textsuperscript{229}. Additionally, AFSCs have some further advantages compared to other foetal MSCs, namely: negligible ethical concerns associated with the cell harvest due to the routine performance of amniocenteses for prenatal diagnostics as well as easy expansion of AFSCs in culture\textsuperscript{219,220,224}.

AFSCs may also be functionally superior to other stem cell types. For instance, despite the similarities between AFSC and adult MSC proteomes, there are a number of proteins associated with cell growth, proliferation, differentiation, angiogenesis and protection against cell death and oxidative stress that are only found in AFSC and not in adult bone marrow MSCs\textsuperscript{230,231}. Additionally, AFSCs were \textasciitilde 40\% more effective than adult bone marrow MSCs in promoting capillary formation \textit{in vivo}\textsuperscript{231}.

In an MI setting, AFSCs were shown to decrease infarct size in a rat model of acute IRI when administered at reperfusion\textsuperscript{232}. Despite the fact that the AFSC engrafted into the myocardium and co-stained for endothelial and vascular smooth muscle cell markers, the conditioned medium from AFSC exhibited similar protective effects\textsuperscript{232,233}. Furthermore, infarct size-limiting and antiapoptotic effects were found after only 2 h into reperfusion suggestive of
acute and not regenerative effects\textsuperscript{232}. Similar findings were reported for amniotic membrane-derived MSCs, which are believed to contribute to the AFSCs population\textsuperscript{227,234}. In addition to the cardioprotective activities, AFSCs can also have proangiogenic effects \textit{in vitro} and \textit{in vivo}\textsuperscript{231}, improve heart function post MI\textsuperscript{190} and even induce myoblast proliferation\textsuperscript{206}. Thus, AFSCs represent a novel alternative to adult MSCs and cardiac progenitor cell populations for treatment of cardiovascular diseases. AFSCs are an attractive and unexplored source of secreted factors, which may have huge clinical potential for the treatment of IRI and its consequences.

At least two populations of AFSCs exist – round-shaped epithelial-like AFSCs (RS-AFSCs) and spindle-shaped fibroblast-like cells (SS-AFSCs)\textsuperscript{219,225,231}. SS-AFSCs represent only a small fraction (~6%) of the AFSCs but they have considerably higher expansion capacity than RS-AFSCs, increased expression of MSC markers and higher migratory capacity \textit{in vitro}\textsuperscript{219}. Yet, it remains unknown which subpopulation of AFSCs will be optimal for use in an IRI setting.

The study of AFSC EVs has only commenced recently, and their functional effects are not well understood and virtually all the studies investigating AFSC-released vesicles were published during the course of this project\textsuperscript{154,229,233,235–239}. Until now, there have been no comparisons between AFSC subtypes (\textit{i.e.} SS-AFSC vs RS-AFSC) and most published articles do not specify the subtype identity of the cells used. From the morphological appearance of the AFSCs in the previously published reports, however, it would appear that the current study is the first one to specifically investigate the more potent SS-AFSC as a source of cardioprotective and proangiogenic sEVs.
Chapter 2. Materials and methods

*Parts of this chapter have been published in the following articles (Ref*\textsuperscript{134,138}):


2.1. Animals, cells and reagents

2.1.1. Animals and reagents

All regulated procedures were approved by the Animal Welfare and Ethical Review Body (AWERB) and were carried out in accordance with Animals (Scientific Procedures) Act 1986 passed by the UK parliament. The author of this thesis and the collaborators (Dr Zhenhe He) are covered by Procedure Individual Licences and the project was conducted under Procedure Project Licence 70/8556 (“Protection of the Ischaemic and Reperfused Myocardium”) issued to Dr Sapna Arjun and covering the Hatter Cardiovascular Institute, UCL. The experiments also followed European guidelines ("Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes").

Male Sprague-Dawley rats were obtained from Charles River UK and used throughout this study at the indicated weights. Food and water were provided *ad libitum* unless stated otherwise.

The reagents used here were obtained from Sigma (later acquired by Merck) unless stated otherwise.
2.1.2. Cell culture

2.1.2.1. Mouse cardiac endothelial cells (MCECs)

Transformed MCECs, derived from microvascular cardiac endothelium of neonatal mice\textsuperscript{240}, were obtained from Cedarlane Cellutions Biosystems. Cells were maintained in monolayers in DMEM (ThermoFisher) supplemented with 25 mM glucose, 4 mM L-glutamine, 50 units/ml penicillin, 50 µg/ml streptomycin and 5% FBS. Cells were grown in a conventional tissue culture incubator at 37°C / 5% CO$_2$ and detached with 0.05% trypsin / 0.53 mM EDTA for passaging.

2.1.2.2. Human umbilical vein endothelial cells (HUVECs)

HUVECs are multiple-donor primary endothelial cells purchased from Lonza (C2519A). Cells were maintained in monolayers in Endothelial Cell Basal Medium 2 (C-22211, PromoCell) supplemented with Endothelial Cell Growth Medium 2 SupplementPack (C-39211, PromoCell) at 37°C / 5% CO$_2$ and passaged using TrypLE Express Enzyme (ThermoFisher). According to the manufacturer, HUVECs maintain endothelial cell identity up to 15 population doublings. Therefore, HUVECs from passages 5-11 were used for experiments.

2.1.2.3. Spindle-shaped amniotic fluid stem cells (SS-AFSCs)

Primary human SS-AFSCs were provided by Dr Pascale Guillot, Institute of Child Health, UCL. The ethical approval to Dr Guillot laboratory was given by the Research Ethics Committees of Hammersmith & Queen Charlotte’s Hospitals (2001/6234) and from NRES Committee London (14/LO/0863) in compliance with the United Kingdom Guidelines for collection of foetal tissue for research\textsuperscript{241}. SS-AFSCs were isolated from second trimester human amniotic fluid in Dr Guillot’s laboratory\textsuperscript{220,242}. Amniotic fluid from a healthy donor was spun to pellet the cells which were resuspended in complete medium, seeded and incubated until the appearance of adherent fibroblast-like cells. Single colonies were dissociated, propagated, pooled and
characterised for MSC markers\textsuperscript{177}. SS-AFSCs were maintained in monolayers in DMEM (ThermoFisher) supplemented with 25 mM glucose, 4 mM GlutaMAX, 50 units/ml penicillin, 50 µg/ml streptomycin and 9% FBS (F9665, Sigma). Cells were grown in a conventional tissue culture incubator at 37°C / 5% CO\textsubscript{2} and detached with TrypLE Express Enzyme (ThermoFisher) for passaging. SS-AFSC from passages 11-20 were used for experiments.

All cell cultures were regularly tested for contamination with \textit{Mycoplasma} species using LookOut Mycoplasma PCR Detection Kit (Sigma) or Mycoplasma Detection Kit (SouthernBiotech).

2.1.2.4. Isolation and culture of primary adult rat ventricular cardiomyocytes (ARVCs)

ARVCs were isolated as described previously\textsuperscript{64} with some modifications. Rats (250-350 g) were anaesthetised with 250 mg/kg pentobarbital (Animalcare). Upon loss of pedal reflex, thoracotomy was performed, heart eviscerated and placed in ice-cold buffer (130 mM NaCl, 5.4 mM KCl (Fisher), 1.4 mM MgCl\textsubscript{2}, 0.4 mM Na\textsubscript{2}HPO\textsubscript{4}, 4.2 mM HEPES, 10 mM glucose, 20 mM taurine (Alfa Aesar) and 10 mM creatine, 0.2 µm-filtered, referred to as “Buffer” below). The aorta was immediately cannulated, and the heart was retrogradely perfused with Buffer supplemented with 750 µM CaCl\textsubscript{2} (Fluka) and maintained at 37°C and pH 7.4 to clear the blood and accommodate the heart to the artificial perfusion system. This was followed by a Ca\textsuperscript{2+} washout period using Buffer containing 100 µM EGTA (a Ca\textsuperscript{2+} chelator) as high Ca\textsuperscript{2+} may induce cell death during digestion phase\textsuperscript{243}. The heart was then digested with Buffer supplemented with 0.016% (w/w) collagenase (Worthington) per 100 g animal weight, and 100 µM CaCl\textsubscript{2} (necessary for optimal collagenase activity). 0.01% (w/w) protease was also added to the digestion buffer for isolations in chapter 8. After perfusion for ~15 min, the ventricles were excised, mechanically disrupted and gently shaken for further ~15 min in an incubator at 37°C. The solution was then sieved to remove fibrotic tissue and debris. Cardiomyocytes were collected by gravity-driven sedimentation or by using very low speed centrifugation (< 100 g, 5 min). Extracellular Ca\textsuperscript{2+} was gradually
restored using consecutive washes with Buffer containing 500 µM and 1 mM CaCl₂ and finally cells were resuspended in Medium 199 (ThermoFisher) supplemented with 5 mM creatine, 2 mM carnitine, 5 mM taurine (Alfa Aesar), 50 units/ml penicillin and 50 µg/ml streptomycin. ARVCs were seeded in individual dishes (chapter 8) or 24-well plates (chapter 5) on areas preincubated for at least 1 hour with ~3-4 µg/cm² laminin to facilitate cell adherence. ARVCs were kept overnight in a conventional tissue culture incubator at 37°C / 5% CO₂ before being used for experiments as indicated.

### 2.2. Isolation of sEVs

#### 2.2.1. Isolation of sEVs from rat blood

1. **Preparation of plasma for ultracentrifugation (UC) or size-exclusion chromatography (SEC)**

Rats (300-400 g) were anaesthetised with 250 mg/kg pentobarbital. Upon loss of pedal reflex, thoracotomy was performed, and blood was collected from inferior vena cava. Syringes used for blood collection were pre-filled with in-house prepared citrate buffer (~15 mM after dilution with blood). Blood samples were centrifuged at 1,600 g for 15 min, room temperature to remove cells. The supernatant plasma was further centrifuged at 10,000 g for 30 min, room temperature to remove platelets, debris and large vesicles and obtain platelet-free plasma (PFP). From this point onwards, PFP was kept on ice. PFP was immediately processed for sEV isolation or frozen at -80°C.

2. **Isolation of plasma sEVs using UC**

An indicated volume of PFP was diluted with PBS to ~7-8 ml and ultracentrifuged for 70 min at 100,000 g, 4°C to pellet the sEVs (polycarbonate tubes, 355630, Beckman Coulter; MLA-55 rotor, Optima MAX-XP, Beckman Coulter). Supernatant was discarded by decanting the tube and sEVs were resuspended in PBS (~7-8 ml) for washing. A second UC run was performed using the same parameters. The sEV pellet was resuspended in 100-200 µl PBS and frozen at -80°C.
2.2.1.3. Isolation of plasma sEVs using SEC

Commercially available qEVoriginal SEC columns (iZON Science, 70 nm cut-off matrix) were used to fractionate PFP according to the manufacturer’s protocol\textsuperscript{160} with some modifications (Figure 2-1). 0.5-1 ml PFP aliquot was loaded on a qEV column and left to enter the column by means of gravity. The column was then flushed with room temperature PBS. The first 3.0 ml of eluent was discarded. Fractions of 0.5 ml were then collected and stored at -80°C.

![Figure 2-1. Principle of size-exclusion chromatography (SEC) for isolation of sEVs](image)

Samples (blood plasma or concentrated cell-conditioned medium) are loaded on top of a SEC column and allowed to enter the column. Collection of eluent fractions (typically 0.5 ml) begins immediately after addition of the samples on the column. Sample constituents separate by gravity inside the column matrix upon addition of PBS: larger particles (e.g. sEVs, yellow/brown circles) elute in early fractions while smaller particles (e.g. soluble protein) enter the pores of the gel matrix inside the column and elute later. Fractions are analysed or used as indicated.
2.2.2. Isolation of sEVs from cell-conditioned culture medium

2.2.2.1. Preparation of conditioned medium for SEC

Conditioned medium was collected from ~5-10 x 10^6 SS-AFSCs (2x T225 flasks, 60 ml culture medium in total) cultured in serum-free conditions for 48 h (24 h time point was also used for initial characterisation experiments). SS-AFSC sEVs used for in vivo experiments (section 2.6.) and endothelial tube formation experiments (section 2.9.) were isolated from ~40-55 x 10^6 SS-AFSCs (10x T225 flasks, 300 ml culture medium in total). Immediately after collection, conditioned medium was spun at 300 g for 10 min, 4°C to remove dead cells. Supernatant was then centrifuged at 10,000 g for 40 min, 4°C (polycarbonate tubes, 355630, Beckman Coulter; MLA-55 rotor, Optima MAX-XP, Beckman Coulter) to remove cell debris and large vesicles. After discarding the pellet, conditioned medium was concentrated to ~200-500 µl using Vivaspin-15R ultrafiltration units (30 kDa, Hydrosart membrane, Sartorius). The remaining concentrate was immediately processed for SEC. See Figure 2-2 for an overview of the procedure.

2.2.2.2. Isolation of sEVs using SEC

Concentrated conditioned medium (≤ 500 µl) was loaded on a qEVoriginal column and processed as described for plasma (section 2.2.1.3.) with some modifications (Figure 2-2). The first 2.5 ml eluent from the column were discarded and fractions of 0.5 ml were then collected and stored at -80°C. After the initial characterisation experiments, 3.5 ml eluent were discarded in the beginning followed by subsequent collection of 1.5 ml (the peak sEV fraction) which was stored at -80°C for analytical and functional experiments.
Figure 2-2. Isolation of sEVs from conditioned cell culture medium using SEC

Conditioned cell culture medium was collected (1) and loaded on ultrafiltration units (Vivaspin, Sartorius) for concentration (2). Ultrafiltration concentrators were spun, the filtrate was discarded, and conditioned medium was topped up on the filters. This was repeated until all of the conditioned medium was loaded on the filters and the final concentrate was ≤ 500 µl. This concentrate was then collected (3) and loaded on a qEVoriginal SEC column (IZON Science) (4). sEV-rich SEC fractions were collected as explained in section 2.2.2.2. and stored as indicated (5). Ultrafiltration device image was taken from https://www.fishersci.co.uk/ and SEC column image was taken from https://www.izon.com/.
2.3. Characterisation of the isolated sEVs

2.3.1. Nanoparticle tracking analysis (NTA)

NTA was used to estimate the concentration of particles in the samples in this report. It is a commonly used technique using light scattering to measure particle concentration and size\textsuperscript{156,244}. Here, NanoSight LM10-HS instrument (Malvern) and Violet (488 nm) laser module were used, together with NTA Software Version 3.1. Particles in the samples were detected under continuous pump flow by scattering of a laser beam light. Brownian motion of each individual particle was analysed by the software and mean squared displacement (\textit{i.e.} displacement of the particle from its original position in a two-dimensional scale) was calculated during acquisition of 3-5 videos of 30-60 s. Temperature (which affects the Brownian motion) was recorded by a probe inserted in the laser module and manually input in the system. NTA software calculates particle sizes using the Stokes-Einstein equation\textsuperscript{244}.

Particle concentration and size was determined using general recommendations\textsuperscript{156} adapted to the type of samples used in these studies. Camera Level of 15 and Detection Threshold of 3-4 was used. Sample concentrations in each experiment were measured by the same operator (\textit{i.e.} the author of this thesis), using the same settings and, if possible, during the same day and using the same sample dilutions to reduce variation to a minimum\textsuperscript{155}.

2.3.2. Protein content

Protein concentrations were measured by standard bicinchoninic acid (BCA) assays or Bradford assays.

BCA assay relies on the reduction of Cu\textsuperscript{2+} to Cu\textsuperscript{+} by the proteins present in the sample\textsuperscript{245}. The Cu\textsuperscript{+} ion forms a stable complex with the BCA which absorbs light at 562 nm\textsuperscript{245}. Due to the low protein concentration in most of the sEV-rich samples, BCA protein assay kit for low concentrations (ab207002, Abcam) was used. During the course of this project, I realised that the BCA assay is not sensitive enough in the lower detection range and it is also prone to interference by various additives, \textit{e.g.} phenol red\textsuperscript{246} or phospholipids\textsuperscript{247}.
(present in conditioned cell culture medium and abundant in sEVs, respectively). Hence, some samples were measured with an in-house made 96-well plate Bradford assay (as indicated in each section). Bradford assay relies on direct binding of Coomassie G-250 blue dye to proteins which increases absorption of the dye at 595 nm\textsuperscript{248}.

Specific details of the procedures are provided in each chapter. Protein content for each experiment was measured using the same assay and, if possible, during the same experiment/on the same plate to minimise variation.

2.3.3. Dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA)

DELFIA is an immunoassay similar to the enzyme-linked immunosorbent assay (ELISA). DELFIA is characterised by high-sensitivity, low background and time-resolved fluorescence detection which all provide a better operational concentration range\textsuperscript{249}. The high sensitivity and the low background of the assay are achieved by substitution of the enzyme-linked reaction in ELISA with a lanthanide element (here europium, Eu, was used). Eu has a very large stokes shift, \textit{i.e.} possesses absorption and emission spectra which do not overlap, allowing excitation at < 400 nm and detecting emission at > 600 nm\textsuperscript{249,250}. This modified immunoassay is suited to analyse samples with small protein quantities such as sEV isolates.

DELFIA has previously been used by Welton \textit{et al.} for sEV marker detection\textsuperscript{159}. This protocol was modified to suit the needs of the current investigation and DELFIA was used to detect protein markers of sEVs or lipoproteins. Aliquots of the samples were diluted to 100 μl with PBS and added directly to the wells of a high-binding 96-well plate (DY990, R&D Systems). Samples were allowed to adhere to the plate overnight at 4°C. The wells were then washed using DELFIA wash buffer (PerkinElmer) and blocking was performed with 100 μl of a 1% (w/v) BSA / PBS solution for 1 h at room temperature. After washing, 100 μl of primary antibodies in PBS were incubated for 2 h at room temperature. This was followed by another wash step and incubation with 100 μl of secondary antibodies in PBS for 1h at room temperature. Details of the primary and secondary antibodies are provided in
each section below. The purchased secondary antibodies were preadsorbed and conjugated to biotin. After a wash step, 100 μl of Eu-labelled streptavidin (100 ng/ml in DELFIA Assay Buffer, PerkinElmer) was added to each well and incubated for 1 h at room temperature. The labelling ratios of the specific lots of streptavidin-Eu conjugate used here were 6.9-7.4 Eu$^{3+}$/streptavidin. After extensive washing, 100 μl of DELFIA Enhancement Solution (PerkinElmer) was added to each well to release the Eu from the antibody complex. The plate was then shaken on a FLUOstar reader (BMG Labtech) at 700 rpm for 5-10 min. Time-resolved fluorimetry was performed on a PHERAstar reader (BMG Labtech) with the following settings: 337 nm excitation, 620 nm detection, 200 μs lag time, 400 μs integration time. Results are presented as arbitrary units of time-resolved fluorescence signal (AU).

2.3.4. Transmission electron microscopy (TEM)

TEM was performed to provide high-resolution images of the contents of conditioned medium and various sEV isolates following previously published procedures with some modifications\(^96\). Assistance with staining and imaging was kindly provided by Mark Turmaine, UCL.

An aliquot (2 μl) of each sample was adsorbed on Formvar/carbon-coated electron microscopy grids. These were then washed with distilled water and stained with 0.5% (w/v) uranyl acetate in H$_2$O at pH 7 for 3-4 min. TEM grids were imaged on a JEOL JEM-1010 electron microscope (JEOL Ltd).

2.3.5. Dot blot protein analysis

SS-AFSC lysate, conditioned medium or isolated SS-AFSC sEVs (conditioned medium and sEVs were lysed using 0.1% (v/v) Triton X-100 and vortexing\(^{251}\)) were pipetted on nitrocellulose membranes (10600003, GE Healthcare). After drying out, the membranes were blocked using a solution of 5% (w/v) BSA / 0.1% Tween-20 in tris-buffered saline (TBS) for 1 h at room temperature and incubated with primary antibodies overnight at 4°C. Secondary antibodies were added for 1 h at room temperature. Membranes
were imaged using Odyssey system (LI-COR). Antibody details can be found in the sections below.

2.3.6. Proteomics: liquid chromatography – tandem mass spectrometry (LC-MS/MS)

Samples for LC-MS/MS analysis were prepared and provided to the UCL proteomics team at the Institute of Women’s Health, UCL (Dr Harvey Johnston and Dr John Timms). The procedure described below was performed by the proteomics team and the protocol was provided by them.

SS-AFSC-conditioned medium or isolated SS-AFSC sEVs containing ~30 µg of protein were lysed using 0.5 % (w/v) sodium dodecyl sulfate and vortexing, and concentrated to ~50 µl on Vivaspin-500 ultrafiltration units (5 kDa cut-off membranes, Sartorius). Samples were then reduced using 200 mM tris(2-carboxyethyl)phosphine for 1 hour at 60˚C, alkylated with 200 mM methyl methanethiosulfonate for 10 minutes at room temperature, and digested overnight with 1 µg of proteomics grade trypsin (Promega). Detergent was removed using a detergent removal column (ThermoFisher) and peptides were isolated and fractionated by solid phase extraction using 100 µl C18 tips eluting into 5, 10, 15, 20, 25, 30, 35, 40 and 60 % acetonitrile in 0.1% ammonium hydroxide. The peptide fractions were lyophilised and resuspended in 0.1% formic acid. ~500 ng of each fraction was loaded by Dionex Ulitmate 3000 (ThermoFisher) onto a Pepmap100 C18 trapping cartridge (5 mm x 5 µM x 0.3 µm) and eluted over a reverse phase gradient (6% (3 min), 35% (35 min), 90% (40 min) organic phase (95% acetonitrile, 5% DMSO, 0.1% formic acid) in aqueous phase (5% DMSO, 0.1% formic acid)). Peptides were resolved with a Pepmap C18 (25 cm x 3 µm x 100 Å) at 300 nl/min and analysed with an LQT-Orbitrap XL mass spectrometer (ThermoFisher). Mass spectrometry (MS) analysis of peptides was performed between 400 and 1700 m/z at 60,000 mass resolution. The top 4 precursor ions per MS scan were characterised by tandem MS with CID (ion trap MS, 2 Da isolation window, 35 keV). The DMSO ion at 401.922718 was used as a lockmass. Target-decoy searching of raw spectral data was conducted with Proteome Discoverer software version 1.4.1.14 (ThermoFisher). Spectra were searched using SequestHT (version
1.1.1.11) against the human and bovine UniProt Swissprot database (downloaded July 2019; supplemented with cRAP contaminations). Settings were: a fragment ion mass tolerance of 0.5 Da, a precursor mass tolerance of 10 ppm, searching for tryptic peptides allowing one missed cleavage, fixed modification of Methythio (C), variable modification of oxidation (M), deamidation (N,Q) with Percolator used to estimate false discovery rate with a threshold of \( q < 0.01 \).

Label-free quantitation (LFQ) enrichment ratios were calculated by LFQ of a 2ppm precursor ion area and were normalised on total protein observation. A minimum LFQ peptide area value (defined as the smallest observed peptide quantitation) was used to replace missing values for calculating enrichment ratios. The minimum value was 1.64E+04 (normalised to 3.53E+05 for SS-AFSC-conditioned medium on total protein observation).

Peptide-spectrum match (PSM) enrichment ratios were calculated by determining the relative number of PSMs observed for each protein after normalisation on total protein observation.

Full data of the identified proteins can be accessed on https://drive.google.com/open?id=1fUyiEZNi_FKIXOCqjBNOBF8VN_kLDa6f. “CM” notation in the table refers to SS-AFSC-conditioned medium, while “sEVs” notation refers to SEC-isolated SS-AFSC sEVs. “sEV-enr > 1.5x” refers to proteins enriched in sEVs > 1.5 times by LFQ peptide area or exclusive to sEVs.

### 2.3.7. Triglyceride content

Triglyceride content of SEC fractions was estimated using a commercially-available triglyceride assay (Cayman Chemical). Triglycerides are mostly present in VLDL and chylomicrons\(^{252}\) and are used here as an indicator of lipid content. The Triglyceride Enzyme Mix used for reaction was prepared in 50 mM phosphate buffer and contained lipoprotein lipase to hydrolyse triglycerides releasing glycerol, glycerol kinase and glycerol phosphate oxidase to release \( \text{H}_2\text{O}_2 \) from glycerol, and peroxidase, 4-aminoantipyrine and \( m \)-anisidine to convert the released \( \text{H}_2\text{O}_2 \) to a purple
quinoneimine product\textsuperscript{253}. Further details about the procedure are provided in section 8.3.6.

2.3.8. EV-TRACK

All experimental data about sEV isolation protocols and characteristics were submitted to the EV-TRACK database (EV-TRACK IDs: EV180051 and EV190058)\textsuperscript{162}. The EV-TRACK database searches (see sections 4.1 and 5.1.) were performed on 11/07/2019.

2.4. sEV uptake studies

These experiments were performed to track isolated sEVs and investigate whether they are taken up into target endothelial cells or cardiomyocytes. The assumptions made by the EV field were that lipophilic dyes can be used to label sEVs by incorporating their lipophilic tails into the EV membranes, similarly to cell membranes, thereby leaving the fluorescent heads to be detected by fluorescence or confocal microscopy\textsuperscript{138,254–260}.

Procedures for sEV labelling with lipophilic dyes and uptake studies are explained in section 8.3.7.

2.5. \textit{In vitro} models of reactive oxygen species- and hypoxia/reoxygenation-induced cell death

2.5.1. Reactive oxygen species (ROS)-induced cell death

A model of hydrogen peroxide (H\textsubscript{2}O\textsubscript{2})-induced cell death was established for assessment of \textit{in vitro} ARVC cell death induced by ROS. In the setting of IRI, excessive ROS production at reperfusion is suggested to contribute to cell death\textsuperscript{24} and therefore protection against ROS-induced cell death can partially mitigate IRI\textsuperscript{55,57}.

ARVCs were isolated and cultured according to section 2.1.2.4 and plated in 24-well plates. Cells were treated with H\textsubscript{2}O\textsubscript{2} at the indicated concentrations for 2 h in medium. Where protective agents were tested, ARVCs were treated for 30 min with N-acetylcysteine (NAC) or SS-AFSC sEVs
prior to H$_2$O$_2$ addition. NAC or SS-AFSC sEVs were present during the H$_2$O$_2$ incubation (see Figures 5-1 and 5-2 for more details). At the end of the treatments, medium was collected and refrigerated, and 1% Triton X-100 in PBS was added for 15 min to lyse the remaining live cells. Media and lysates were immediately processed for lactate dehydrogenase assay (section 2.5.3.). Untreated ARVCs were used as a control.

Further details about the procedure can be found in section 5.3.1.

2.5.2. Hypoxia/reoxygenation-induced cell death (simulated IRI in vitro)

2.5.2.1. Cells and composition of the buffers

A model of hypoxia/reoxygenation of ARVCs in vitro, previously established in the lab$^{64}$, was used to simulate IRI. ARVCs were isolated and cultured according to section 2.1.2.4 and plated in 24-well plates. Cells were incubated with hypoxic and normoxic buffers mimicking normal and ischaemic milieu found in the heart during MI. The composition of the buffers is provided in Table 2-1.

<table>
<thead>
<tr>
<th>Table 2-1. Composition of normoxic and hypoxic buffers used in simulated IRI experiments in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normoxic Buffer (pH 7.4 at 5% CO$_2$)</strong></td>
</tr>
<tr>
<td><strong>Substrate</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Glucose</td>
</tr>
<tr>
<td>NaCl</td>
</tr>
<tr>
<td>KCl</td>
</tr>
<tr>
<td>KH$_2$PO$_4$ (Fisher)</td>
</tr>
<tr>
<td>MgSO$_4$ x 7H$_2$O (Acros Organics)</td>
</tr>
<tr>
<td>NaHCO$_3$ (Fisher)</td>
</tr>
<tr>
<td>CaCl$_2$ (Fluka)</td>
</tr>
<tr>
<td><strong>Hypoxic Buffer (pH 6.4 at 5% CO$_2$)</strong></td>
</tr>
<tr>
<td><strong>Substrate</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>NaCl</td>
</tr>
<tr>
<td>KCl</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
</tr>
<tr>
<td>MgSO$_4$ x 7H$_2$O</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
</tr>
<tr>
<td>CaCl$_2$</td>
</tr>
</tbody>
</table>
2.5.2.2. Experimental procedure

In groups subjected to hypoxia, cells were washed, and culture medium was replaced with Hypoxic Buffer. Plates were transferred to a modular incubator chamber (Billups-Rothenberg) which was purged with 5% CO₂ / 95% N₂ to achieve hypoxic environment. The chamber was sealed and placed into an incubator at 37°C for 5 h. Hypoxic Buffer was then collected and refrigerated while cells were reoxygenated for 1 h in Normoxic Buffer to mimic reperfusion (Reoxygenation Buffer). At the end of the experiment, Reoxygenation Buffer was collected and refrigerated. 1% Triton X-100 in PBS was added for 15 min to lyse the remaining live cells. Hypoxic Buffer, Reoxygenation Buffer and cell lysates were immediately processed for lactate dehydrogenase release assay (section 2.5.3.). A control group with ARVCs incubated in Normoxic Buffer was included.

Further details about the procedure and treatments can be found in section 5.3.2.

2.5.3. Lactate dehydrogenase (LDH) assay

LDH release was used as a surrogate measurement for cell death. LDH is a cytoplasmic enzyme released in the extracellular milieu upon disruption of the cell membrane. The assay relies on the conversion of 4-iodonitrotetrazolium violet to a purple, 490 nm light-absorbing formazan product in the presence of LDH.

LDH was assayed using Pierce LDH Cytotoxicity Assay Kit (ThermoFisher) following the manufacturer’s instructions with some modifications. 50 µl of each collected sample (25 µl diluted to 50 µl in the case of ROS-induced cell death experiments due to higher cell number) were pipetted on a 96-well plate. 50 µl assay buffer were added per well and the plate was shaken for 15 min protected from light. LDH Stop Solution was added and the absorbance at 490 nm was read on a FLUOstar plate reader (BMG Labtech). LDH release was expressed as a percentage of total LDH.
2.6. *In vivo* model of non-recovery IRI in rats

*In vivo* experiments were performed by Dr Zhenhe He at the Hatter Cardiovascular Institute, UCL. Experiments were designed, and samples were prepared by the author of this thesis. The study was randomised, and the operator was blinded to the administered treatment. Data analysis was performed by Dr Zhenhe He and the author of this thesis, and the mean value was taken on all occasions.

2.6.1. Anaesthesia, ventilation and preparation for the procedure

Rats (230-320 g) were anaesthetised with an initial dose of 100 mg/kg pentobarbital. After loss of pedal reflex, animals were placed in a supine position on a heat mat. Limbs were secured with tape and a rectal probe was inserted to monitor body temperature and keep it constant at 36.5-37.5°C. Tracheostomy was performed, and artificial ventilation was achieved by means of a modified human Abbocath-T intravenous 16 G catheter (Smiths Medical International) connected to a Small Animal Ventilator (Harvard Apparatus). Respiratory rate was set to 75 breaths/min, tidal volume was 8-9 ml/kg and expiratory tube was submerged in water to apply 2 cmH$_2$O positive end-expiratory pressure. 1-lead electrocardiogram (ECG) was recorded using PowerLab 4/30 system (AD Instruments) and LabChart 7 software (Figure 2-3A-C). Jugular vein was cannulated using tubing attached to a 21 G butterfly needle for intravenous administration of substances.

2.6.2. Induction of myocardial infarction

Chest fur was removed with a hair clipper and a commercially available hair-removal cream. A skin incision was made laterally on the left side, ~1 cm below the forelimbs and perpendicular to the sternum. Thoracotomy was performed by blunt dissection of pectoral muscles. Incision was made at the 4th intercostal space, the ribs were retracted, and the heart exposed. After tearing the pericardium, 9.3mm 3/8 circular round-bodied needle was used to insert a 6-0 braided silk suture underneath the left anterior descending (LAD)
coronary artery. Two 6-0 braided silk suture loops were placed on each arm of the suture to facilitate subsequent removal, and the suture was tightened. The presence of myocardial ischaemia was confirmed by myocardial blanching distal to the suture and ECG changes (the latter are not always evident) (Figure 2-3A-C). Treatments were administered intravenously into the jugular vein, 2 min prior to reperfusion as a single bolus dose (500 µl/animal, see section 5.3.3. for details of the treatments). After 30 min of ischaemia, reperfusion was achieved by releasing the suture, which remained in place for subsequent staining. The chest was then closed, and the ischaemic myocardial territory was reperfused for 2 h. See Figure 2-4A for an overview of the procedure.

Figure 2-3. ECG changes during in vivo ischaemia/reperfusion in rats
A – ECG of a rat before the start of the MI procedure. B – ECG changes during ischaemia – QRS complex broadening and ST-segment depression (left, typically early in ischaemia); ST-segment elevation (right, typically later in ischaemia). C – ECG changes during reperfusion – QRS complex broadening (left, in early reperfusion); ST-segment elevation (right, in late reperfusion). Note that these changes are not always visible during the procedure. This was found to be due to the variables beyond the operator’s control including the presence of metal tools (e.g. chest retractors, needle holders etc.), the decompression of the chest cavity upon surgical opening and the positioning of the suture inside the myocardium.
2.6.3. Heart collection and staining

At the end of the protocol, the rib cage was opened, and the heart excised. It was swiftly cannulated and washed with PBS to clear the blood. The LAD artery was reoccluded and ~1 ml Evans Blue dye (1% (w/v) in PBS) was injected through the cannula into the coronaries to demarcate the non-at-risk area (Evans-Blue-positive) and area-at-risk (AAR) (Evans-Blue-negative) (Figure 2-4B). The heart was removed from the cannula, wrapped in cling film and briefly frozen.

For infarct size (IS) measurements, the heart was removed from the freezer and cut in transverse plane into 5-6 segments. Slices were stained with triphenyl tetrazolium chloride (TTC; 1% (w/v) in phosphate buffer at pH 7.4) at 37°C for 15 min. Heart segments were then transferred to a 3.7% (w/v) formaldehyde solution and left at room temperature overnight for fixation. 2D images were obtained the next day using a CanoScan LiDE 220 scanner (Cannon).

2.6.4. Area-at-risk (AAR) and infarct size (IS) measurements

ImageJ software was used to demarcate and calculate the area of the non-at-risk tissue (defined as Evans-Blue-positive), the AAR (defined as Evans-Blue-negative) and the infarct area (defined as Evans-Blue-negative and TTC-negative). AAR was presented as a percentage of the total area of the left ventricle. IS was defined as the percentage of infarct within the AAR (Figure 2-4C).

35 rats were used in total. 17 animals died during the experimental procedure (49%): 13 died prior to or unrelated to the administered treatments, 4 died post injection (2 in the Vehicle group, 1 in the Bradykinin group, and 1 in the sEV group). Hence, treatment-related death was not found in this experiment.
Figure 2-4. *In vivo* model of non-recovery IRI in rats

A – Left panel: Overview of the anaesthetised animal during the procedure (during the ischaemic period). The animal was anaesthetised and positioned on a heat mat. The ECG leads (yellow – ECG electrodes, blue – ground electrode) and rectal temperature probe (on the left) are visible. Right panel: The heart of the animal during the ischaemic period. Note, the rib cage is retracted to expose the heart and the suture is tied. B – Evans Blue staining of the heart. The blue area represents the non-at-risk heart tissue, the non-stained area represents the area-at-risk (AAR). The tightened suture is visible on the left-hand side on the dividing line (dotted white line). C – Heart slice stained with TTC. Non-at-risk area is visible as Evans-Blue-positive, the AAR is visible on the left of the dotted yellow line and it is Evans-Blue-negative. TTC staining of the AAR demarcates the viable tissue in red and the non-viable tissue (*i.e.* infarct) in white (demarcated with a white dotted line).
2.7. Endothelial cell migration assays

Promigratory functions of SS-AFSC sEVs on HUVECs were investigated using a modified Boyden’s Chamber migration assay\textsuperscript{262}. 12-well NeuroProbe chemotaxis chamber (AA12, NeuroProbe) was used for all migration experiments (Figure 2-5A). Chemoattractants or putative chemoattractants diluted in PBS were pipetted in the bottom compartment of the chamber at the indicated concentrations, while 30,000 HUVECs per well were added at the top compartment in Endothelial Serum-Free Defined Medium (Cell Applications Inc; 113-500, Sigma). Inclusion of pathway inhibitors or antibodies to the migration experiments was achieved by their addition to both top and bottom compartments at the indicated concentrations. The two compartments were separated by polycarbonate track-etch membrane with 8-µm pores (PFB8, NeuroProbe) allowing HUVECs to transmigrate to the bottom part only actively (Figure 2-5A-C). The cell number was selected such that there is no overfilling: the well area is 18 mm\(^2\) and there are 1,000 pores/mm\(^2\) resulting in 1,667 cells/mm\(^2\). Since pores occupy \(\sim\)5\% of the filter area, 1 of 12 cells will be likely to randomly settle over a pore of the membrane resulting in \(\leq\) 8\% of the cells being likely to transmigrate to the bottom compartment solely based on their localisation over a membrane pore. Incubation timings published in the literature varied widely\textsuperscript{263–266} and after a pilot characterisation experiment to establish the maintenance of a concentration gradient of macromolecules across the membrane, a 6-h incubation (at 37°C / 5% CO\(_2\), see sections 6.3.1. and 7.3.3.) was chosen. The incubation was followed by membrane collection and removal of the non-migrated cells from the top side of the membrane using a manufacturer provided rubber wiper (NeuroProbe). Membranes were immediately fixed in 100% ice-cold methanol for 5 min followed by staining using 0.5 % (w/v) Crystal Violet solution in 20% methanol. Membranes were washed in PBS from the excess dye and scanned on CanoScan LiDE 220 scanner (Cannon). To avoid bias in selecting fields for analysis, mean staining intensity of each well as a whole was quantified using ImageJ (Figure 2-5B,C). Background intensity was measured for each membrane and subtracted from the well intensity values. The majority of the experiments were performed in duplicates (unless
the number of the experimental groups exceeded the number of available chamber wells for duplicates).

Details of chemoattractants and inhibitors are provided in each section.

---

**Figure 2-5. Modified Boyden’s Chamber migration assay setup and analysis**

A modified Boyden’s Chamber migration assay was used in this report to investigate the migration of HUVECs in response to SS-AFSC sEVs. A – Left panel: 12-Well Chemotaxis Chamber (NeuroProbe) used in the experiments. Right panel: bottom wells of the chemotaxis chamber were filled with the testing substance (e.g. vehicle or SS-AFSC sEVs depicted) and a porous membrane was added on top (dotted red line). HUVECs were added in the top wells and chambers were incubated for 6 h. B – Membranes were collected, cells from the top side of the membrane were scraped (non-migrated cells), and crystal violet staining was performed. Membranes were scanned on a conventional scanner and images were analysed using ImageJ. Representative examples shown of a vehicle (left) and SS-AFSC sEV sample (right). C – A close-up microscopic image of the stained membranes. Membrane pores are indicated by yellow arrows. Black objects are migrated cells (or clusters of migrated cells). Scale: 40 µm.
2.8. Endothelial cell proliferation assays

HUVEC proliferation was assayed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This assay detects the conversion of the yellow MTT dye by cellular mitochondrial reductases to a purple formazan product which absorbs light at 570 nm thus reflecting the metabolic activity of the cells providing an indication of the cell number\textsuperscript{267}.

5,000 HUVECs/well were plated on a 96-well plate (~15,000 cells/cm\textsuperscript{2}) in Endothelial Cell Basal Medium 2 (C-22211, PromoCell) supplemented with Endothelial Cell Growth Medium 2 SupplementPack (C-39211, PromoCell). Cells were allowed to attach for 2 h at 37°C / 5% CO\textsubscript{2} followed by washing and replacement of medium with Endothelial Serum-Free Defined Medium (Cell Applications Inc.; 113-500, Sigma). Cells were incubated for further 48 h at 37°C / 5% CO\textsubscript{2}, exchanging the medium once at 24 h. After the incubation period, MTT at 0.45 mg/ml final concentration in PBS was added to each well. Plates were kept at 37°C / 5% CO\textsubscript{2} for further 3 h to allow for formation of formazan crystals from the MTT in the living cells\textsuperscript{267}. This was followed by removal of the supernatant, addition of lysis solution (0.1 M HCl / 10% Triton X-100 in propan-2-ol) and shaking for 10 min at 300 rpm. The absorbance at 570 nm (formazan) and 690 nm (background) was measured on a FLUOstar plate reader (BMG Labtech). The results are presented as A\textsubscript{570}-A\textsubscript{690}.

2.9. Endothelial cell tube formation assays

HUVEC tube formation assay on extracellular matrix-mimicking gel was conducted to investigate for proangiogenic effects of SS-AFSC sEVs \textit{in vitro}\textsuperscript{268,269}. The assay performed here is an optimised version developed by Dr Bishop-Bailey’s group which uses a thin-layer extracellular gel matrix (i.e. Geltrex\textsuperscript{™}, ThermoFisher)\textsuperscript{270}. It is aimed at a significant reduction of resources and an optimised application for microscopy studies\textsuperscript{270}. Protocol and guidance on performing the experiments were kindly provided by Prof Caroline Wheeler-Jones and Dr Elizabeth Finding from Royal Veterinary College.

Geltrex\textsuperscript{™} was thawed at 4°C and kept on ice during pipetting. 2 µl gel was pipetted in each well of a 96-well plate. The gel was evenly distributed in
the well by the use of an insert of a tip for a repeater pipette. This was performed on ice to minimise gel solidification due to exposure to higher temperatures. The plate was then transferred to a cell culture incubator at 37°C for ~1 h to ensure Geltrex™ is completely solidified.

HUVECs were starved for 1-1.5 h in Endothelial Cell Basal Medium 2 (PromoCell) supplemented with 1% FBS (without growth factors to eliminate interference with tube formation). Cells were then collected, resuspended and pipetted into wells covered with Geltrex™ at 10,000 cells/well. Treatments were added at the indicated concentrations and plates were incubated for 16 h in the incubator at 37°C / 5% CO₂. After the incubation period, wells were washed with PBS and fixed using 4% (w/v) formaldehyde solution. Images were taken using Nikon Eclipse TE200 inverted microscope (Nikon). Tube-like structures were manually counted using ImageJ software.

2.10. Flow cytometry

For SS-AFSC characterisation, SS-AFSCs were collected and pelleted for 5 min at 500 g. Supernatant was removed and cells were washed once with 1% (w/v) BSA in PBS. Cells were then resuspended in 50 µl 1% (w/v) BSA in PBS containing primary antibodies (see section 4.3.2. for further details) and samples incubated on ice for 1 h in dark. If required, cells were washed and incubated with secondary antibody (1:50) for 1 h in dark (on ice). Cells were then pelleted, washed once and resuspended in 300-500 µl 1% (w/v) BSA in PBS for flow cytometry. 10,000 events were counted for each sample excluding debris using BD Accuri™ C6 flow cytometer (BD Biosciences).

Cell death and apoptosis of SS-AFSCs were detected using Apoptosis Detection Kit I (556547, BD Biosciences) following the manufacturer’s instructions. The kit incorporates propidium iodide staining (to detect dead cells, i.e. cells with compromised membranes, by binding of propidium iodide to the nuclear DNA) and annexin V labelling (to detect apoptotic cells by binding of annexin V to phosphatidylserine phospholipid heads, exposed at the outer plasma membrane leaflet during apoptosis²⁷¹). SS-AFSCs were collected at the indicated time points and washed two times with ice-cold PBS. Cells were resuspended in Annexin V Binding Buffer (containing high Ca²⁺
necessary for annexin V binding to phosphatidyl serine) and Annexin V and PI were added (both at 1:20). The mixture was incubated for 15 min at room temperature in dark. Annexin V Binding Buffer was then added to a final volume of 300-500 µl for flow cytometry. 10,000 events were counted for each sample excluding debris BD Accuri™ C6 flow cytometer (BD Biosciences). Single propidium iodide or annexin V staining was used for signal compensation calculated by FlowJo Version X software.

2.11. Western blotting

2.11.1. Conventional Western blotting

HUVECs were pre-starved for 3 h in DMEM (ThermoFisher) supplemented with 25 mM glucose, 4 mM GlutaMAX, 50 units/ml penicillin (Sigma), 50 µg/ml streptomycin (Sigma). Cells were then treated for 15 min with vehicle (PBS), insulin (positive control at 100 nM or 500 nM as indicated; I9278, Sigma) or SS-AFSC sEVs (as indicated). GDC-0941 (100 nM, SM19-10, Cell Guidance Systems), a PI3K-specific inhibitor272, was added 10 min prior to administration of vehicle/sEVs. Following the treatments, cells were immediately lysed in situ using a buffer consisting of 100 mM Tris base, 300 mM NaCl and 0.5% NP-40, pH 7.4 with added protease inhibitors (1861279, ThermoFisher) and phosphatase inhibitors (78427, ThermoFisher). Debris was discarded by 10 min centrifugation at 10,000 g and the protein-rich supernatant was stored at -80°C.

20 µg protein (as measured by a BCA assay) of each sample was loaded in wells of a 10% Tris-Glycine pre-cast gels (XP00105BOX, ThermoFisher). This was followed by electrophoresis using 25 mM Tris base / 250 mM glycine / 0.1% (w/v) SDS buffer and wet transfer of proteins to nitrocellulose membranes (10600003, GE Healthcare). The membranes were then blocked using a solution of 5% (w/v) BSA in PBS and incubated with primary antibodies at 1/1,000 dilution in 5% (w/v) BSA / 0.1% Tween-20 in PBS overnight at 4°C. Secondary antibodies were added at 1/10,000 dilution in 1 : 1 ratio Odyssey Blocking Buffer (LI-COR) : PBS and incubated for 1 h at room temperature. Membranes were imaged, and densitometry was performed on Odyssey
system (LI-COR). Results are presented relative to 500 nM insulin positive control or GAPDH internal reference.

### 2.11.2. Wes™ Simple Western

Wes™ Simple Western (ProteinSimple) is an immunoprobing system designed to integrate sample loading, protein separation, immunoprobing and detection *in situ*. The procedure requires Wes™ apparatus and ready-to-use manufacturer-provided cartridges and reagents. It allows probing of samples with very low protein quantities in a time-sparing procedure with a large dynamic range of detection.

Manufacturer’s procedure was followed for Wes™ Simple Western. Sample protein was denatured using manufacturer’s provided buffer (DTT-based) and loaded on Wes™ multiwell plates. Cartridges with a set number of capillaries provided by the manufacturer and containing the gel required for protein separation were loaded on the Wes™ apparatus. The samples were automatically drawn inside the capillary gels and protein separation, blocking, antibody recognition, horseradish peroxidase reactions, washing and detection were sequentially and automatically performed on the Wes™ machine. Primary antibodies were the only reagents which were not provided by the manufacturer and their identities are listed below (see section 3.3.4.). Anti-mouse (042-205, ProteinSimple) and anti-rabbit (042-205, ProteinSimple) secondary antibodies conjugated to horseradish peroxidase were used according to the manufacturer’s instructions. Images were acquired and analysed using Compass software for Simple Western (ProteinSimple).

### 2.12. Protein arrays

Protein profiling of SS-AFSC-conditioned medium or SS-AFSC sEVs was performed using Proteome Profiler Human Angiogenesis Array Kit and Cytokine Array Kit (ARY007 and ARY005B, respectively; R&D Systems). Phosphorylation of intracellular signalling kinase pathways was investigated using Proteome Profiler Human Phospho-Kinase Array Kit (ARY003B, R&D Systems).
Manufacturer’s instructions were followed with some modifications as indicated in section 7.3.1.

2.13. Statistical analysis and display items

Sample size is stated in figure legends and data are plotted as single values or means ± SEM. Statistical analysis were performed using unpaired Student’s t-tests, 1-way or 2-way ANOVA with post-hoc tests (indicated in figure legends). Pearson’s or Spearman’s correlation tests were performed where indicated after a Kolmogorov-Smirnov test for normality. Data were analysed, and graphs were produced using GraphPad Prism 5.0 (GraphPad Software). $\alpha$ was set at 0.05, i.e. $p < 0.05$ was deemed statistically significant.

Servier Medical Art (SMART) was used to produce some of the figures in this thesis under the Creative Commons Attribution 3.0 Unported License (https://smart.servier.com/).
Chapter 3. Comparison of SEC and UC for blood plasma sEV isolation

This chapter has been published as a standalone article (Ref 134):


3.1. Introduction

Blood-derived sEVs have recently been described as cardioprotective64,89,169,273 and proangiogenic149,274 nanoparticles. Blood plasma and serum are rich sources of sEVs133,159 but also contain very high concentration of protein133,145 and other nanoparticles such as lipoproteins140,148,159. Soluble protein and lipoproteins are frequently co-isolated with sEVs from blood with virtually every known sEV isolation method133,140,141,144. There have been some recent reports indicating that it may in fact be impossible to separate plasma proteins145 and lipoproteins140 from vesicles due to a direct physical association of the contaminants with the sEVs. Multiple attempts to compare techniques for isolation of sEVs have been made133,143,145,148,159,161,275,276 but there is no agreement in the field for an optimal procedure and specific protocols. A possible reason for this is the lack of thorough systematic comparative studies. This is evidenced by querying the EV-TRACK database to obtain an EV-METRIC score (reflecting the amount of reported information about the procedures and the isolated EVs162, see section 2.3.8.). This score is generally very low for blood plasma EV studies and a striking 29% of all 170 submitted experiments scored 0%, indicating that none of the essential information about the protocols or EV identity was provided. This is a clear indication of a lack of transparency in reporting in the blood plasma EV field.

Concerns about technique and procedural optimisation, however, is not solely limited to experiments using blood as a source of EVs. As discussed in section 1.6.2., it was reported that nearly 50% of the published experiments
investigating cell-culture conditioned medium-derived sEVs were performed in the presence of serum\textsuperscript{109}. Considering that lipoproteins in plasma and serum are abundant\textsuperscript{139}, together with the presence of ample soluble non-EV protein in serum (e.g. albumin), this may result in a significant lipoprotein and protein contamination that can interfere with downstream experiments.

Multiple issues may originate from these impurities. For instance, true sEV numbers may be overestimated due to the inability of enumeration techniques to discriminate between vesicles and protein or lipoprotein aggregates\textsuperscript{140}. This will inevitably confound functional studies using treatments normalised to particle number. Another problem may arise with the presence of co-isolates such as growth factors and HDL which possess cardioprotective properties\textsuperscript{277,278} and can produce false positive results in studies investigating the sEV role in IRI.

Some commonly used sEV purification methods rely only on concentration of the EVs rendering the samples highly impure (e.g. precipitation\textsuperscript{148,157}), while others provide better sEV purity but potentially physically damage the vesicles, yield considerably fewer sEVs or involve laborious procedures while still being unable to separate completely sEVs from lipoproteins or soluble proteins (e.g. ultracentrifugation\textsuperscript{140,145,148,279}, density gradient centrifugation\textsuperscript{141} or immunocapture\textsuperscript{146}). In fact, some of the least pure methods (i.e. precipitation) were used in the majority of the recent studies investigating the cardioprotective effects of sEVs isolated from promising populations of CDCs (see section 1.6.3.)\textsuperscript{67,68,196,280}.

It has been proposed that size-exclusion chromatography (SEC) can be used to eliminate many of the aforementioned confounders. This is a technique that preserves vesicular morphology and function, does not require expensive equipment and is performed in considerably less time\textsuperscript{145,157,159–161}. However, it is currently known that SEC also suffers from contamination with lipoproteins (especially APOB\textsuperscript{+} lipoproteins)\textsuperscript{138,140,142,159}.

In this chapter, SEC was compared to the most-commonly used and well-established differential ultracentrifugation (UC)\textsuperscript{96,109} for isolation of blood plasma sEVs. This was intended to provide an indication of lipoprotein and protein contamination of the samples in case of use of blood products, such
as serum, in cell cultures (chapter 4-7), but also to evaluate SEC as emerging technique for sEV purification from blood for functional or biomarker studies.

3.2. Aims

The aims of this chapter were to:

1) Compare SEC to UC for isolation of blood plasma sEVs
2) Systematically characterise the obtained sEVs for protein content, particle number, sEV-specific markers and presence of contaminating factors
3) Evaluate the compatibility of blood products with SEC for future use in experiments for purification of sEVs from conditioned cell culture medium
3.3. Methods

3.3.1. Blood plasma sEV isolation

For UC, 1 ml PFP aliquot was diluted with PBS to ~7-8 ml and ultracentrifuged. Pellets were resuspended to final volumes of ~230 µl.

For SEC, 1 ml PFP aliquot was loaded on the qEVoriginal column for sEV isolation and fractions 3.5 – 7.5 ml were collected and analysed (except for the pilot experiment where fractions 1.0 – 18.0 ml were collected and analysed). Where indicated, peak sEV fractions were pooled and concentrated using Vivaspin-4 (100 kDa, polyethersulfone membrane) filter (Sartorius).

Protein, particle and tetraspanin content were normalised to volumes as indicated in the figures.

3.3.2. Protein content

Protein content was estimated by BCA protein assay for low concentrations (ab207002, Abcam) according to the manufacturer’s instructions. Reaction Mixture was prepared at a ratio of 25 : 25 : 1 of Reagent A : Reagent B : Reagent C. Sample aliquots were diluted to 150 µl with PBS and mixed with 150 µl Reaction Mixture in 96-well plates. The reactions were incubated for 120 min at 37°C and absorbance at 562 nm was measured on a FLUOstar plate reader (BMG Labtech). Protein concentration was calculated using BSA standards and a 4-parameter logistic curve.

3.3.3. Dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA)

Information about the antibodies used in this chapter is provided in Table 3-1. Primary antibodies were used at 1 µg/ml in PBS and secondary antibodies at 0.25 µg/ml in PBS.
Table 3-1. Antibodies used for DELFIA studies

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Species / Antibody type</th>
<th>Clone / Cat no</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD9</td>
<td>Mouse IgG1</td>
<td>M-L13</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD81</td>
<td>Mouse IgG1</td>
<td>JS-81</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>HSP70</td>
<td>Mouse IgG1</td>
<td>N27F3-4</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>APOB</td>
<td>Rabbit IgG</td>
<td>H-300</td>
<td>Abcam</td>
</tr>
<tr>
<td>Mouse IgG1</td>
<td>Goat</td>
<td>ab98691</td>
<td>Abcam</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>Goat</td>
<td>ab97073</td>
<td>Abcam</td>
</tr>
</tbody>
</table>

3.3.4. Wes™ Simple Western

Wes™ Simple Western was used as a confirmatory experiment to show HSP70 and APOB presence in as well as ACTN4 absence of the indicated isolates. ~0.4-0.6 µg protein/lane was used. The following antibodies were used: HSP70 (at 10 μg/ml; clone N27F3-4, Santa Cruz Biotechnology), APOB (at 20 μg/ml; clone H-300, Santa Cruz Biotechnology), ACTN4 (at 32 μg/ml; clone C2C3, GeneTex).
3.4. Results

3.4.1. Protein and particle content of sEV samples isolated by SEC or UC of blood plasma

In this section, the quantity of contaminants in the sEV isolates obtained by SEC of blood plasma was assessed. SEC was directly compared to a well-established and commonly used method for sEV isolation – UC. PFP samples were obtained after clearance of cells and debris using sequential centrifugation steps and according to published recommendations. Equal volumes (1 ml) of blood PFP were processed for SEC and UC for direct comparison of the two techniques (Figure 3-1A).

Pilot SEC experiments were performed to identify the sEV-rich eluent fractions and soluble protein-rich ones. sEV tetraspanin markers eluted with a peak at ~5-6 ml, while the bulk of contaminating soluble protein eluted with a peak at ~10 ml (Figure 3-1B). Therefore, fractions 3.5 ml – 7.5 ml were chosen for subsequent collection to analyse sEV yields and purity.

As expected, protein amount increased from fraction 3.5 ml to fraction 7.5 ml (Figure 3-2A) but surprisingly, particle number also followed this increase without a clear early peak (Figure 3-2B). Particle/protein ratio, as a marker of sEV purity, peaked at 5.0 ml and 5.5 ml suggesting particle enrichment in these fractions which matched the pilot sEV tetraspanin peak (Figure 3-1B, 3-2C). Therefore, SEC fractions 5.0-5.5 ml were deemed the fractions richest in sEVs.

Particle number, protein content and particle/protein ratios in the sEV samples isolated by UC were considerably lower in comparison to SEC fractions 5.0-5.5 ml (Figure 3-2A,B). Particles were similarly distributed in terms of size for UC and SEC fractions 5.0-5.5 ml (Figure 3-2D) but the modal size was higher in UC samples compared to SEC fractions 5.0-5.5 ml (UC: 96.6 ± 3.1 nm vs SEC: 81.5 ± 3.3 nm, p < 0.05) (Figure 3-2E).

Overall, protein and particle content of the sEV samples isolated by SEC and UC indicated a greater yield of particles with an apparent better purity for SEC in comparison to UC.
Figure 3-1. Pilot data from SEC fractionation of rat blood plasma

A – Rat blood was collected from the inferior vena cava of anaesthetised animals. After removal of cells and large debris by centrifugation, equal aliquots (1 ml) were processed for sEV isolation by SEC (qEVoriginal column, iZON Science) or UC. B – Data from a pilot SEC experiment. Rat PFP was loaded on a SEC column and eluent fractions were collected (1.0 ml – 18.0 ml). Protein content (black line) and sEV-specific tetraspanin markers (CD9, green; CD81, blue; HSP70, red) were assayed for each collected eluent fraction by BCA assays or DELFIA, respectively. A peak of sEV-rich SEC fractions was detected at 5.0 ml – 6.0 ml (shaded on the graph) while the bulk of the soluble protein eluted with a peak at 10.0 ml.
Figure 3-2. Protein content and particle numbers of sEV isolates of rat blood plasma subjected to SEC or UC

Rat PFP pre-cleared from cells and large debris was subjected to SEC or UC. A – Amount of protein in SEC fractions 3.5 ml – 7.5 ml and UC samples measured by a BCA assay. n = 6. B – Number of particles in SEC fractions 3.5 ml – 7.5 ml and UC samples measured by NTA. n = 6. C – Ratio of particle number to protein content for SEC fractions 3.5 ml – 7.5 ml and UC samples (particles/µg protein). n = 6. D – Size-distribution of particles in SEC fractions 5.0 ml – 5.5 ml and UC samples measured by NTA. Lines represent mean of n = 6-12. E – Modal size of the particles in SEC fractions 5.0 ml – 5.5 ml and UC samples measured by NTA. * p < 0.05; Student’s t-test. n = 6-12.
3.4.2. Yield and purity of the sEV samples obtained by SEC or UC of blood plasma

Measurements of particulate content and protein amount are frequently not sufficient determinants of the purity or yield of the harvested sEV isolates\textsuperscript{118}. Therefore, the samples were further characterised by DELFIA (an immunoassay with high sensitivity and improved detection range\textsuperscript{249,250}, see section 2.3.3. for more information) for sEV-specific protein markers (CD81 and HSP70\textsuperscript{64,98,116}) and a lipoprotein-specific marker (APOB\textsuperscript{139,140,161}). CD81\textsuperscript{+} and HSP70\textsuperscript{+} particles were detected in the UC samples, but the signal was higher in the SEC isolates with a peak at 5.5 ml, coinciding with the particle/protein ratio peak (Figure 3-3A,B). There was a strong positive correlation between CD81 and HSP70 content of the peak sEV fractions indicative of a high specificity of the assay (Figure 3-3C). Despite the greater particle yields of SEC, normalisation of the CD81 and HSP70 signal to the total protein content showed similar or higher enrichment of sEV-specific markers in UC samples (Figure 3-3D). This suggested that SEC samples may have higher soluble protein contamination, although this was similar to UC for the peak sEV fraction (fraction 5.5 ml).

To specifically assess the lipoprotein contamination, APOB levels in the SEC and UC samples were assayed. APOB\textsuperscript{+} lipoproteins eluted from the SEC column in a very similar pattern to sEVs, an indication of significant lipoprotein contamination in SEC isolates (Figure 3-4A). An APOB signal was detectable in the UC samples but it was \~80 times lower than the peak sEV fraction from SEC (fraction 5.5 ml) (Figure 3-4A). Additionally, normalisation of APOB signal to total protein content, showed \~30 times greater enrichment of APOB in SEC fraction 5.5 ml in comparison to UC samples (Figure 3-4B).

Immunoblotting confirmed that sEV-specific proteins are higher in UC samples and also verified the absence of a large- and medium-sized EV marker ACTN4 from the sEV isolates\textsuperscript{116} (Figure 3-4C). Strikingly, it further showed that APOB signal is higher in SEC samples compared to UC ones and also in pooled early SEC fractions compared to the starting plasma sample, effectively showing that SEC can be used to enrich for APOB\textsuperscript{+} lipoproteins (Figure 3-4C). Additionally, when these pooled SEC fractions were
concentrated using an ultrafiltration unit, a macroscopically opaque appearance of the concentrate further suggested the presence of a very high amount of lipids (Figure 3-4D).
Figure 3-3. sEV-specific markers in samples of rat blood plasma subjected to SEC or UC

Rat PFP pre-cleared from cells and large debris was subjected to SEC or UC. Samples were assayed for sEV-specific markers (CD81, HSP70) using DELFIA. A – CD81 content in SEC fractions 4.0 ml – 7.0 ml and UC samples. AU – arbitrary units (volume normalisation performed). n = 6. B – HSP70 content in SEC fractions 4.0 ml – 7.0 ml and UC samples. AU – arbitrary units (volume normalisation performed). n = 6. C – Correlation of HSP70 and CD81 content in SEC fractions 4.0 ml – 7.0 ml and UC samples. p < 0.0001; Spearman’s correlation test, Spearman r = 0.958. n = 48. D – CD81 or HSP70 signal from panels A and B normalised to the total protein content for sEV-rich SEC fractions 5.0 ml – 6.0 ml and UC samples. n = 6.
Figure 3-4. APOB, sEV markers and medium and large EV markers in the sEV isolates of rat blood plasma subjected to SEC or UC

Rat PFP pre-cleared from cells and large debris was subjected to SEC or UC. Samples were assayed for lipoprotein-specific marker (APOB) using DELFIA. A – APOB content in SEC fractions 4.0 ml – 7.0 ml and UC samples. AU – arbitrary units (volume normalisation performed). n = 6. B – APOB signal from panel A normalised to the total protein content for sEV-rich SEC fractions 5.0 ml – 6.0 ml and UC samples. n = 6. C – Wes™ immunoblotting for HSP70 (sEV marker, top), ACTN4 (medium- and large-sized EV marker, middle) and APOB (lipoprotein marker, bottom). SEC samples used were from pooled 3.5 ml – 6.0 ml fractions. Two different pooled SEC samples (SEC 1 and SEC 2) and UC samples (UC 1 and UC 2) were used for APOB probing. Note the presence of ACTN4 in plasma but not in sEV samples and the marked enrichment of APOB in SEC samples compared to the starting plasma. D – Representative images of the macroscopic appearance of a pooled SEC 3.5 ml – 6.0 ml fraction sample and an UC sample.
TEM images supported the data obtained with NTA and DELFIA. SEC fraction 4.0 ml, containing low sEV and lipoprotein markers, was mostly void, with only a few large lipoprotein-resembling structures (Figure 3-5). SEC fraction 5.5 ml, rich in sEVs but also in lipoproteins, showed the presence of concave sEV-like discs but also lipoprotein-like elements (Figure 3-5). Finally, dense, aggregated material was visible in the soluble protein-rich fraction 8.0 ml (Figure 3-5). UC samples had mostly sEV-like structures with some lipoproteins visible as well (Figure 3-5).

Figure 3-5. TEM images of sEV isolates of rat blood plasma subjected to SEC or UC
Transmission electron micrographs of SEC fractions 4.0 ml, 5.5 ml and 8.0 ml (top), and UC samples (bottom). Arrows: particles resembling lipoproteins (electron-lucent, white appearance). Arrowheads: particles resembling sEVs (with typical erythrocyte-like cup shapes). Scale bars: 200 nm.
The findings above regarding sEV yield and purity from soluble proteins and lipoproteins are summarised in Figure 3-6A-C. Overall, UC harvested only ~50% of the sEVs obtained in the richest sEV SEC fraction 5.5 ml with similar soluble protein contamination (albeit slightly lower for the UC samples) and a striking ~94% lower lipoprotein contamination.

Taken together this strongly suggests that SEC may provide increased sEV yield from plasma but prominently reduced sEV purity compared to UC, especially with regard to the contaminating lipoproteins. This is a clear indication of the interference of lipoproteins with SEC for sEV isolation.

Figure 3-6. Yield and purity of sEV isolates of rat blood plasma subjected to SEC or UC

Key data from Figures 3-2, 3-3 and 3-4 summarised. A – sEV yield for sEV-rich SEC fractions 5.0 ml – 6.0 ml and UC samples (represented by CD81 content). B – Protein contamination of sEV-rich SEC fractions 5.0 ml – 6.0 ml and UC samples (represented as µg protein/CD81 signal ratio). C – Lipoprotein contamination of sEV-rich SEC fractions 5.0 ml – 6.0 ml and UC samples (represented by APOB signal/CD81 signal ratio).
3.5. Discussion

In this chapter the yields and purities of sEV samples obtained by SEC of blood plasma were investigated and compared to the commonly employed UC approach for harvesting of sEVs. Results suggested that SEC isolates plasma sEVs with a better yield, but compromised purity compared to UC which was mainly due to the marked presence of contaminating lipoproteins. This further demonstrated the need to assay specific markers of sEVs and contaminants (e.g. APOB) in characterisation experiments since parameters such as particle number and protein content were not sufficient as standalone indicators of vesicular purity. It is noteworthy, that the experiments presented here received high EV-METRIC scores of 62-66%, which place them in the top 4% of all experiments with blood plasma sEVs to date in the EV-TRACK database (EV-TRACK entry ID: EV180051).

We have previously proposed that a pure population of ~1x10^{10} sEVs contains ~1 µg of total protein in theory. In fact, other authors used cell culture bioreactor systems for large scale sEV production and showed that a ratio of 3x10^{10} particles/µg protein can be achieved in high purity samples (discussed in more detail in section 4.5.) Strikingly, the ratio that the same authors were able to obtain for blood serum samples was an order of magnitude lower at < 2x10^{9} particles/µg protein. Similar ratio was obtained in this chapter in blood plasma UC samples. On the contrary, sEV-rich samples of SEC had high particle/protein ratios, reaching high purity values of 1.5-2x10^{10} particles/µg protein. These apparent ratios, however, were found to be masked by the contaminant factors, predominantly by the presence of co-isolated APOB+ lipoproteins. Note that this is not likely to be specific to NTA, since other techniques used to obtain vesicle counts, such as tunable resistive pulse sensing (TRPS), have also been shown to readily detect lipoproteins in the sEV samples. Nevertheless, some techniques, such as bead-capture followed by flow cytometry, may provide further useful information and distinguish better the vesicles from contaminants. Obtaining an accurate count of the sEVs in the samples is of extreme importance as particle concentration is the parameter chosen by many authors to be reported in functional experiments which can lead to severe discrepancies in the literature.
Hence, particle and protein parameters of an sEV sample are not useful as sole indicators of sEV yield, and particle/protein ratio is not sufficient to provide detailed information about the purity of an sEV isolate. Our immunoassay data for sEV and lipoprotein markers indicated that the particles in the early SEC fractions were mostly non-sEV material, likely lipoproteins. The levels of APOB signal in sEV-rich SEC fractions were extremely high and the APOB/CD81 signal ratio was on average ~60 times higher in the richest sEV SEC fraction compared to the UC samples. This is in accordance with the findings of others who used the same commercial SEC columns as the ones employed in the current report (qEVoriginal by iZON Science)\textsuperscript{140}, but also with authors using manually prepared SEC columns\textsuperscript{159}. In fact, the co-isolation of lipoproteins is expected as some of the most abundant APOB\textsuperscript{+} classes of lipoproteins have remarkable similarities with sEVs with regard to their size (see Figure 1-3 and supporting information in the main text). Furthermore, it has previously been suggested that ¾ of the nanosized particles detected in plasma are not sEVs\textsuperscript{159}. In fact, more recent reports indicated that blood plasma lipoproteins outnumber the sEVs by orders of magnitude (\~10\textsuperscript{16} lipoproteins/ml)\textsuperscript{139}. Interestingly, even density gradient centrifugation, an isolation technique considered to be of the highest purity, is unable to purify sEVs from APOB\textsuperscript{+} particulate material\textsuperscript{140} and due to similar densities, co-isolates HDL with sEVs\textsuperscript{141}. Thus, there is an inherent inability of the currently used techniques to separate lipoproteins from sEVs. This may be solved by employing a combination of multiple purification techniques\textsuperscript{109,142}, although at the expense of reduced yields and increased variability due to the multistep procedures. This approach may be particularly useful to separate lipoproteins when using SEC columns of sufficient length and other SEC matrices such as Sephacryl\textsuperscript{283}, or a column matrix combining different isolation principles such as vesicle binding to the matrix and sEV size separation\textsuperscript{284}. Alternatively, newer methods such as asymmetric field flow fractionation have also shown to reduce lipoprotein contamination to minimum (< 10% of the nanoparticle-associated proteins)\textsuperscript{98} but they are not widely available due to the costly equipment requirements and laborious procedures involved.

Another study by Baranyai \textit{et al.} compared purities of rat blood plasma sEVs isolated by SEC or UC\textsuperscript{145}. Similar to the findings here, authors reported
marked protein impurities in the sEV isolates but they argued that SEC provides better sEV purity when compared to different UC protocols with regard to contamination with albumin\textsuperscript{145}. Despite that, even by using a 12 times larger SEC column, the authors were unable to completely separate sEVs from albumin\textsuperscript{145}. This indicates that abundant blood proteins may severely interfere with purification of sEVs when using SEC. Therefore, in a broader perspective it may be advisable that blood product-free medium is used for studies with tissue or cell cultures.

This study has the advantage of providing a direct systematic comparison of SEC and UC since plasma samples from the same rat and equal volumes for SEC and UC isolations were used, which was not employed by previous reports\textsuperscript{145}. Here, determination of total protein content estimated that SEC sEVs can also be contaminated with varying amounts of soluble protein exceeding that of UC for some SEC fractions. In fact, the peak-sEV SEC fraction and UC isolates contained similar sEV marker signal relative to protein suggestive of comparable soluble protein contamination in both techniques.

It is noteworthy that SEC elution profile may depend on the origin of the starting sample. For instance, it was shown here that sEVs elute with a peak at 5.5 ml when using plasma. This is delayed in comparison to peaks observed with conditioned medium that tend to appear earlier, \textit{e.g.} \textasciitilde4.5 ml (see chapter 4). This is likely to be due to the much higher viscosity in the plasma samples leading to bigger overlap of the eluting fractions (\textit{i.e.} sEVs and bulk protein overlap more in this instance). Taking this into account and since fraction collection is an operator-dependent procedure, analysis of the whole profile of early eluent fractions is desirable to account for sample- and operator-dependent variations and to identify the sEV-rich fractions.

Notably, particles isolated by UC were larger than particles in the sEV-rich SEC fractions. This has at least two possible explanations. Firstly, UC might induce particle aggregation or fusion as a result of the high centrifugation speeds\textsuperscript{279}. Alternatively, some of the co-isolated lipoproteins in the SEC fractions are expected to be considerably smaller (\textit{e.g.} LDL\textsuperscript{140}) and their presence in large numbers can reduce the modal size of the particles in these samples. Thus, contamination can also be reflected in a discrepant modal size of the samples. The exact reason for this significant difference in modal size
remains unidentified. Additional confirmatory approaches can be taken, e.g. by TEM sizing of the particles in the samples\textsuperscript{285}. It should be noted, however, that this procedure is error-prone due to potential deformation of the vesicles (i.e. the typical erythrocyte-like appearance), dehydration of the sample and a non-uniform adhesion of the sEVs to the TEM grids as well as variable staining\textsuperscript{285}.

In this chapter, 1 ml of PFP was used for sEV isolation by SEC. 1 ml is the amount of serum contained within 10 ml of tissue or cell culture medium supplemented with 10% FBS. In experiments with cell-conditioned medium, much larger volumes are typically processed for sEV isolation\textsuperscript{109} which requires concentration of the samples prior to SEC. Lipoproteins, but also soluble protein\textsuperscript{138} (see chapter 8), from the serum will be concentrated together with the cell-derived sEVs on the ultrafiltration units. This can lead to a substantial presence of serum-derived particulate and protein material in the sEV harvests. Despite the findings for contaminating lipoproteins, protein contamination of the peak-sEV SEC fraction was only modestly higher in SEC samples compared to the UC isolates. Therefore, the purity of an sEV sample isolated from starting material devoid of lipoproteins and abundant soluble protein, e.g. serum-free conditioned medium, may be greatly improved with SEC application. This is indeed evidenced by a study showing that EVs with higher yields and better purity can be isolated by SEC compared to UC of neuroblastoma Opti-MEM™ serum-free cell-conditioned medium\textsuperscript{275}. Moreover, this can also be translated to an improved functionality as demonstrated for CPC-derived sEVs isolated by SEC of serum-free medium which were more potent in activating intracellular ERK1/2 than sEVs isolated by UC\textsuperscript{276}.

The search for a method to obtain cardioprotective nanoparticles with the highest purity, potency and efficacy continues and here it was demonstrated that blood may not be the optimal source for these. Blood-derived sEVs are co-isolated with large amounts of lipoproteins and may also contain significant amounts of protein. In fact, even superior techniques such as SEC co-purify APOB\textsuperscript{+} lipoproteins and render the final sEV isolate largely impure.

Apart from the importance of these findings for selecting the optimal method for sEV isolation from plasma, data presented in this chapter have
broader implications for research using sEVs isolated from samples containing blood products such as serum. In the next chapters stem cell cultures were used as a source of sEVs with superior purity and lower heterogeneity, exploiting the advantages of SEC to isolate highly-pure, morphologically intact and functionally uncompromised sEVs in a serum-free environment.
Chapter 4. Isolation and characterisation of SS-AFSC sEVs

Data from chapters 4-7 have been published in the following abstract (Ref 286):


Data from chapters 4-7 have also been submitted as a standalone manuscript:

Takov K, He Z, Johnston H, Timms JF, Guillot PV, Yellon DM, Davidson SM. Small extracellular vesicles secreted from amniotic fluid stem cells possess cardioprotective and promigratory potential.

4.1. Introduction

The study of AFSC EVs has only commenced recently, and their functional effects are not well understood154,229,233,235–239. AFSCs are foetal MSCs and, as discussed above (see section 1.7.), they are believed to be more potent cardioprotective, proangiogenic and reparative source than adult MSCs224. Yet, it remains unknown whether AFSC sEVs hold better potential than adult MSCs as therapeutics in the setting of MI.

Culturing cells for sEV collection is a very popular approach in studies of the activities of stem cell secretomes. As an indication of the quality of the studies with conditioned medium-derived sEVs performed to date, a query of the EV-TRACK database was performed162 (see section 2.3.8. for details about EV-TRACK) which returned 22% of all 1215 experiments with conditioned cell culture medium scoring 0% on the EV-METRIC, indicative of the lack of any EV characteristics or experimental parameters in the report. Only 14% scored ≥ 50% – a clear indication that improvements in the transparency and reporting are needed.

Prior to assessing the functional activity of AFSC sEVs, robust characterisation is required to validate the yields and purity of the isolated vesicles. To date, the majority of the AFSC sEV studies do not adhere to
published recommendations for the use of standardised protocols for sEV isolation, and comprehensive characterisation of the obtained sEV samples\textsuperscript{235,236}. Some of the previous experiments use precipitation techniques that harvest ample amounts of contaminants (see section 1.6.2.)\textsuperscript{229,235,237} while others employ extremely long (\(\geq 12\) h)\textsuperscript{238,239} or excessively high-speed (200,000 g)\textsuperscript{236} ultracentrifugation protocols which may compromise sEV integrity leading to vesicle breakdown, aggregation or fusion altering the functions of the harvested sEVs\textsuperscript{279}. In addition to that, culture conditions used previously were not optimal in many investigations: supplementation with EV-depleted FBS was evident in some studies without consideration for contaminating lipoproteins and abundant soluble proteins\textsuperscript{238,239}, one investigation even used FBS not depleted of EVs for culture of the AFSCs, thus co-isolating FBS sEVs together with the AFSC sEVs\textsuperscript{235}, while another group incubated the AFSCs for 24 h in neat PBS for sEV isolation not taking into account the cell death that will occur in these conditions and the accumulation of debris and apoptotic bodies\textsuperscript{236}.

Producing and collecting large amounts of sEVs is a laborious process requiring ample resources and time. Optimising conditions to increase the purity of the obtained sEVs is desirable but studies are rarely focused on this issue. Previous reports indicated that culture of progenitor cells in higher starting cell numbers and for longer incubation times can increase the yield of sEVs without greatly impacting the total protein content, \textit{i.e.} rendering the harvested sEV sample more pure. However, extended incubation can also induce significant cell death\textsuperscript{206} which may result in excessive release of cell debris masking the presence of actively secreted sEVs. Therefore, this fine balance between incubation time, sEV yield and purity requires detailed analysis and characterisation.

Together with the choice of culture conditions, selection of the method for sEV isolation is of utmost importance. SEC is deemed to be one of the best approaches to purify sEVs from the rest of the components in the conditioned cell culture medium, yet with the downside of co-harvesting lipoproteins in cases of serum supplementation of the culture medium (discussed in chapter 3 and shown by us\textsuperscript{134,138} and others\textsuperscript{140,142,145,159}). In serum-free environment, however, the potential of SEC to isolate sEVs with higher yields, better purity
and improved functional efficacy may be fully realised. In addition to that, the previous chapter showed that SEC can isolate higher yields of sEVs in comparison to other techniques (i.e. ultracentrifugation) which can be valuable for maximising the sEV harvest without the need for a bigger starting cell number, thus reducing the required resources. Moreover, SEC preserves vesicular morphology better\textsuperscript{145,157,159–161} and may even preserve vesicular functional activity better\textsuperscript{276}. Taking all these into account, SEC was chosen as the preferred method for isolation of SS-AFSC sEVs here.

During this project two studies by Bollini’s group were published showing the use of standardised ultracentrifugation protocols to isolate sEVs from serum-free AFSC-conditioned medium\textsuperscript{154,233} but only one of them characterised the obtained sEVs\textsuperscript{154}. Additionally, the authors did not identify the subtype of AFSCs used in their reports but the microscopical images provided show cell morphology more reminiscent of the less potent RS-AFSCs (see section 1.7. for AFSC subtype discussion)\textsuperscript{154}. There are currently no published reports on the isolation and activities of sEVs from the more potent SS-AFSCs.

In this section serum-free medium was used for SS-AFSC culture to eliminate confounding contaminants and isolate highly-pure sEVs from the potent subpopulation of SS-AFSCs.

4.2. Aims

The aims of this chapter were to:

1) Establish the identity of the SS-AFSCs and assess their MSC marker expression and cell death after 24 h or 48 h of serum-free culture

2) Isolate and systematically characterise SS-AFSC sEVs after serum-free culture for 24 h and 48 h
4.3. Methods

4.3.1. SS-AFSC serum-free culture

For characterisation and sEV isolation experiments, SS-AFSCs were washed three times with PBS to remove any residual FBS, and serum-free medium was added for 24 h or 48 h. After the incubation period, conditioned medium was processed for sEV isolation and the SS-AFSCs were harvested and counted for normalisation purposes.

SS-AFSCs images were taken using Nikon Eclipse TE200 inverted microscope (Nikon).

4.3.2. Flow cytometry

The primary antibodies used for SS-AFSC characterisation by flow cytometry (at 1:25-1:10 dilution) are provided in Table 4-1.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Conjugate</th>
<th>Clone / Cat no</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14</td>
<td>PE</td>
<td>TÜK4</td>
<td>Miltenyi Biotec</td>
</tr>
<tr>
<td>CD29</td>
<td>PE</td>
<td>MAR4</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD34</td>
<td>FITC</td>
<td>581</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD44</td>
<td>-</td>
<td>J-173</td>
<td>Abcam</td>
</tr>
<tr>
<td>CD45</td>
<td>FITC</td>
<td>HI30</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD73</td>
<td>PE</td>
<td>AD2</td>
<td>Miltenyi Biotec</td>
</tr>
<tr>
<td>CD90</td>
<td>APC-Vio770</td>
<td>DG3</td>
<td>Miltenyi Biotec</td>
</tr>
<tr>
<td>CD105</td>
<td>FITC</td>
<td>43A4E1</td>
<td>Miltenyi Biotec</td>
</tr>
<tr>
<td>Mouse IgG κ chain</td>
<td>PE</td>
<td>IC002P</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>Mouse IgG</td>
<td>Alexa Fluor 488</td>
<td>ab150117</td>
<td>Abcam</td>
</tr>
</tbody>
</table>
4.3.3. SS-AFSC sEV isolation

For SEC isolation, fractions 3.0 – 13.0 ml were collected and analysed. sEV-rich fractions 4.0 ml, 4.5 ml and 5.0 ml were pooled, analysed and referred to as “SS-AFSC sEVs”.

Protein, particle and tetraspanin content were normalised to volumes as indicated in the figures.

4.3.4. Nanoparticle tracking analysis (NTA)

In addition to the previously explained NTA methods, particle concentration was also measured in some samples of 1) starting conditioned medium: after clearing the dead cells and debris with 300 g and 10,000 g centrifugation, and 2) concentrated conditioned medium: after ultrafiltration, i.e. just prior to SEC. See Figure 2-2 for details. Samples were diluted and processed as explained in section 2.3.1.

4.3.5. Protein content

Protein content was estimated by BCA protein assay for low concentrations (ab207002, Abcam) as explained in section 3.3.2., or by Bradford assays. For Bradford assays, 50 µg/ml Coomassie G 250 dye solution was prepared in 5% (v/v) methanol / 8.5% H₃PO₄. Sample aliquots were diluted to 150 µl and mixed with 150 µl of the Coomassie solution on a 96-well plate. The plate was shaken and incubated for 10 min at room temperature before measuring the absorbance at 595 nm on a FLUOstar plate reader (BMG Labtech). Protein concentrations were calculated using BSA standards and a 4-parameter logistic curve.

4.3.6. Dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA)

Antibodies used in this chapter are provided in Table 4-2. Primary antibodies were used at 1 µg/ml in PBS and secondary antibodies at 0.25 µg/ml in PBS.
Table 4-2. Antibodies used for DELFIA studies

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Species / Antibody type</th>
<th>Clone / Cat no</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>1ary</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD9</td>
<td>Mouse IgG1</td>
<td>M-L13</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD63</td>
<td>Mouse IgG1</td>
<td>H5C6</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD81</td>
<td>Mouse IgG1</td>
<td>JS-81</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>2ary</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse IgG1</td>
<td>Goat</td>
<td>ab98691</td>
<td>Abcam</td>
</tr>
</tbody>
</table>

4.3.7. Dot blot protein analysis

~50 ng protein of each sample was lysed and pipetted on nitrocellulose membranes. The following antibodies were used: primary (all at 1 µg/ml in 5% (w/v) BSA / 0.1% Tween-20 / TBS) – CD63 Clone H5C6, BD Biosciences; CD81 Clone JS-81, BD Biosciences; ACTN4 Clone C2C3, GeneTex; secondary (all at 0.05 µg/ml in 1 : 1 ratio of Odyssey Blocking Buffer (LI-COR) : TBS) – goat anti-mouse IgG for CD63 and CD81, 926-32210, LI-COR and goat anti-rabbit IgG for ACTN4, 926-32211, LI-COR.

4.3.8. Proteomics

See section 2.3.6. for details of the LC-MS/MS procedure.

Functional enrichment analysis was performed using g:Profiler (version e96_eg43_p13_563554d, database updated on 05/06/2019) with g:SCS multiple testing correction method and a significance threshold of 0.05\(^{287}\). Proteins detected in the sEV samples were used, excluding those assigned a minimum LFQ value (see section 2.3.6.) and those with < 2 PSMs. Fold enrichment analysis was performed, which represents the occurrence of the proteins associated with the indicated terms relative to the expected occurrence based on the query size and the total human proteome size. Complete analysis can be found on https://drive.google.com/open?id=1fUyiEZNi_FKIXOCqiBNOBF8VN_kLDa6f.

For analysis of bovine proteome contamination, proteins assigned a minimum LFQ value (for both conditioned medium and sEV isolates) and those with < 2 PSMs (for both conditioned medium and sEV isolates) were excluded.
4.4. Results

4.4.1. SS-AFSC characterisation after 24 h or 48 h of serum-free culture

SS-AFSCs used in this and the following sections were harvested and thoroughly characterised for differentiation potential in Dr Pascale Guillot’s laboratory. To further confirm their identity for the experiments presented here, flow cytometry for markers suggested to satisfy criteria for MSC identification was performed. SS-AFSCs were confirmed as CD73+, CD90+, CD105+, CD29+ and CD44+ as well as CD14−, CD34− and CD45− (Figure 4-1).

Serum-free culture is expected to significantly reduce subsequent protein and lipoprotein contamination of the sEV isolates (see chapter 3.). To assess SS-AFSC morphological and phenotypic alterations in serum-free conditions, cells were deprived of FBS for 24 h or 48 h. SS-AFSCs showed morphological elongation in serum-free conditions (Figure 4-2A) which has already been reported for MSCs before. As expected, cells ceased to proliferate in absence of serum (p < 0.05 for 48 h incubation, Figure 4-2B), but numbers did not decrease below the baseline levels even after 48 h (Figure 4-2B). Propidium iodide and annexin-V staining demonstrated acceptable levels of cell survival (> 85% propidium iodide−ve cells) and apoptosis (< 15% propidium iodide−ve, annexin-V+ve cells) for both serum-free conditions (Figure 4-2C-E). It is noteworthy that no differences in cell death between 24 h or 48 h serum-free incubation were evident, despite the slight reduction in cell survival after 48 h of serum-free culture compared to serum-supplemented culture (Figure 4-2D,E). This amount of cell death is considered acceptable as it was not exceeding previously reported values for other AFSC subtypes.

To investigate the retention of marker expression of SS-AFSC under these conditions, flow cytometry for the main MSC surface proteins was performed on cells incubated for 24 h or 48 h in serum-free medium. SS-AFSCs retained expression of CD73, CD90 and CD105 and remained negative for CD34 and CD45 (Figure 4-3A,B). Notably, a slight reduction in CD105 was found as previously reported for MSCs cultured in serum-free conditions (Figure 4-3A). The apparent slight rightward shift of CD34 and
CD45 peaks after 48 h incubation was found to be due to a higher autofluorescence of the cells in this condition (Figure 4-3B,C).

Overall, 24 h or 48 h serum-free incubation had subtle effects on SS-AFSCs viability and MSC marker expression as reported before for other subtypes of AFSCs\textsuperscript{154}. Importantly, only minor changes were present between 24 h or 48 h of serum-free culture.

**Figure 4-1. Characterisation of SS-AFSCs by flow cytometry**

SS-AFSCs were analysed for MSC membrane marker expression using flow cytometry. Grey peaks – unlabelled cells; red peaks – cells labelled for the indicated antigens. Note that cells are largely positive for CD73, CD90, CD105, CD29 and CD44, and negative for CD14, CD34 and CD45.
Figure 4-2. Numbers and cell death of SS-AFSCs incubated with or without serum

SS-AFSCs were incubated for 24 h or 48 h in serum (FBS)-supplemented or serum-free (No FBS) medium. A – Representative images of SS-AFSC morphology in each condition. Scale: 200 µm. B – Cell number after each incubation timing normalised to Day 1. n = 3. C – Propidium iodide and annexin-V double staining of SS-AFSCs for each condition followed by flow cytometry quantification. Single staining compensation applied using FlowJo. Representative plots shown. D,E – Results from C presented graphically. D – Live cells (propidium iodide-negative) as a percentage of total. n = 3. E – Apoptotic cells (propidium iodide-negative/annexin-V-positive) as a percentage of total. n = 3. * p < 0.05, ns – non-significant (p > 0.05), 1-way Repeated Measures ANOVA with Tukey’s post-hoc test.
Figure 4-3. SS-AFSC expression of membrane markers after incubation with or without serum

SS-AFSCs were analysed for membrane marker expression using flow cytometry. A,B – Membrane markers present on (panel A) or absent of (panel B) SS-AFSCs (indicated below graphs) after incubation in serum (FBS)-supplemented (red peaks) and serum-free (No FBS) (green peaks) conditions. Top panels show 24 h incubation and bottom panels show 48 h incubation. Grey peaks show unlabelled cells in 24 h FBS-supplemented condition. C – Unlabelled cells after incubation in FBS-supplemented (grey peak) or No-FBS (red peak) medium for 48 h. Note the slight rightward shift in the background fluorescence after incubation in serum-free medium.
4.4.2. Isolation of SS-AFSC sEVs after 24 h or 48 h of serum-free culture of SS-AFSCs

To isolate SS-AFSC sEVs, ultrafiltration combined with SEC of serum-free conditioned medium was performed after 24 h or 48 h of incubation. SEC easily resolved particles, with a peak elution at 4.0-4.5 ml, from the soluble protein, with a peak elution at 10.5 ml (Figure 4-4A). DELFIA confirmed the presence of sEV-specific tetraspanins (CD9, CD63, CD81) in early SEC fractions with a peak at 4.5 ml (Figure 4-4B,C). Importantly, the amount of protein in the early sEV-rich fractions was very low and the particle peak coincided with an early protein peak (Figure 4-4A,D). The early SEC fractions contained more particles (p < 0.01), protein (p < 0.001) and sEV marker levels (p < 0.01 for CD9, p < 0.001 for CD63 and CD81) after 48 h compared to 24 h incubation (Figure 4-4A-D). Furthermore, sEV purity was greater after 48 h than 24 h culture, as determined by the ratio of CD9 (p < 0.05), CD63 (p < 0.001) or CD81 (p < 0.01) markers to total protein content (Figure 4-4E-G). Finally, the particle/protein ratio for peak sEV fractions was also higher for 48 h incubation, reaching > 1.2x10^{10} particles/µg protein, further indicating the higher purity of the obtained sEVs after longer culture incubation (p < 0.05, Figure 4-4H).

Overall, 48 h serum-free incubation of SS-AFSCs provided increased yield and purity of the obtained vesicles. To minimise soluble protein contamination for further analytical and functional experiments, pooled sEV-rich fractions 4.0 ml, 4.5 ml and 5.0 ml after 48 h incubation were used, hereafter labelled as “SS-AFSC sEVs”.


A  SEC fractionation: particles & protein

B  Tetraspanin sEV markers: 24 h

C  Tetraspanin sEV markers: 48 h

D  Protein amount: early fractions

E  CD9 per µg protein

F  CD63 per µg protein

G  CD81 per µg protein

H  Particle/protein ratio

*Legend on the next page*
Figure 4-4. Isolation of SS-AFSC sEVs using SEC and characterisation of the isolates (see the previous page)

sEVs were isolated by SEC of serum-free medium conditioned by SS-AFSCs for 24 h or 48 h. A – Particle and protein levels of SEC fractions measured by NTA and BCA assays, respectively. p < 0.01 for Particles 24 h vs Particles 48 h. B,C – sEV-specific tetraspanin markers of SEC fractions obtained from 24 h (B) or 48 h (C) incubation of SS-AFSCs measured by DELFIA. p < 0.01 for CD9; p < 0.001 for CD63 and CD81. D – Protein amount of early SEC fractions. Note the low protein quantities and the early protein peak. p < 0.001. E-G – CD9 (E), CD63 (F) and CD81 (G) signal of SEC fractions normalised to protein amount. p < 0.05 for panel E; p < 0.01 for panel G; p < 0.001 for panel F. H – Particle/protein ratio of early SEC fractions. p < 0.05. n = 3 where error bars are present and n = 1 where absent. Curves compared by 2-way Repeated Measures ANOVA (points with n = 3 included).

4.4.3. Comprehensive characterisation of SS-AFSC sEVs

SS-AFSC sEV purification was very effective as ~99.5% of the contaminating soluble protein was removed. SS-AFSCs secreted on average ~1.2 ± 0.5 x10^10 sEVs (~0.9 ± 0.2 µg protein) per 10^6 cells for the 48-h incubation period (Figure 4-5A,B). Particle/protein ratios were mostly above the threshold of ~1x10^10 particles/µg protein suggested to represent a pure population of sEVs, with a mean of 1.7 ± 0.2 x10^10 particles/µg protein (Figure 4-5C). The size-distribution profiles were typical for sEV samples, the majority of the vesicles exhibiting sizes of ≤ 200 nm with a modal size of 100.5 nm (Figure 4-5D). Confirmatory NTA measurements of PBS, used as diluent for sEVs for NTA showed that background particles accounted for < 0.2% of the particles in the SS-AFSC sEV isolates (Figure 4-5E).

Intriguingly, considerable particle loss was observed throughout the isolation process. Conditioned medium was harvested and concentrated on ultrafiltration units before being loaded onto a SEC column. NTA analysis showed 10.3 ± 0.8 x10^4 particles/cell in the starting conditioned medium, 4.0 ± 0.7 x10^4 particles/cell in the concentrated conditioned medium and 1.4 ± 0.1 x10^4 particles/cell in the isolated SS-AFSC sEVs (Figure 4-5F).

Purified SS-AFSC sEVs expressed all characteristic tetraspanin exosome markers with the CD63 signal being the highest and CD9 – the lowest (Figure 4-6A). To further confirm the enrichment of sEVs in the isolates compared to the starting conditioned medium, a dot blot of sEV-specific and
non-s-EV markers was performed\textsuperscript{116}. There was a marked enrichment of CD63 and CD81 in the SS-AFSC sEV samples compared to conditioned medium, and depletion of alpha-actinin-4 (ACTN4), a characteristic protein of medium and large EVs\textsuperscript{116}, in the purified sEVs (Figure 4-6B). Transmission electron microscopy confirmed the presence of large numbers of sEVs with characteristic concave disc shapes\textsuperscript{88} (Figure 4-6C). The purity of the isolated SS-AFSC sEVs was greatly improved relative to the starting conditioned medium in which impurities were clearly visible as aggregated dense material (Figure 4-6C). To definitively determine the constituents of the SS-AFSC sEV samples, aliquots were provided to the UCL Proteomics Team (Institute of Women’s Health, UCL) and analysed using LC-MS/MS by Dr Harvey Johnston and Dr John Timms. Proteomic analysis was performed by the author as indicated. SS-AFSC sEVs were found to be enriched in multiple EV markers\textsuperscript{116} including: CD9, CD63, CD81, syntentin-1, TSG101, ALIX, ADAM10, flotillins, annexins, CD47, CD90 (MSC EV-specific), NT5E, CAV1. Furthermore, they were depleted or devoid of non-s-EV markers\textsuperscript{116} including: HSP90B1 and HSPA5 (endoplasmic reticulum-associated proteins); GOLGA2 (Golgi-associated protein); LMNA (nuclear protein); KRT18 (cytoplasmic protein); and ACTN4 (a marker of medium and large EVs, but not sEVs). Finally, the SS-AFSC sEV proteome was highly enriched in Gene Ontology Cellular Component terms associated with extracellular vesicles and exosomes (p < 1x10\textsuperscript{-150}, Figure 4-6D,E; full table with proteomics data can be accessed on https://drive.google.com/open?id=1fUyiEZNi_FKiXOCqiBNOBF8VN_kLDa6f). Notably, of the proteins that were not expected to co-purify with the sEVs\textsuperscript{118}, core nucleosomal histones (H2A, H2B, H3 and H4) were found to be either exclusive for the sEV fraction or enriched in the sEV fraction compared to the conditioned medium.

A small number (~50) of bovine proteins could be detected in SS-AFSC-conditioned medium and SS-AFSC sEVs, which may be explained by carryover of serum components to the serum-free culture or the similarity of the protein sequences of some bovine and human proteins (Table 4-3). Notably, APOB and other apolipoproteins were not detected, and bovine albumin was depleted from the sEV isolate in comparison to the starting conditioned medium (Table 4-3).
Figure 4-5. Characterisation of pooled sEV-rich SS-AFSC SEC fractions: particle number, protein content and particle recovery

sEVs were isolated by SEC of serum-free medium conditioned by SS-AFSCs for 48 h and sEV-rich fractions 4.0 ml, 4.5 ml and 5.0 ml were pooled and analysed. A – Particle number released by 1 million cells measured by NTA. \( n = 27 \). B – sEV protein content released by 1 million cells measured by Bradford assays. \( n = 10 \). C – Particle/protein ratio. \( n = 13 \). Dotted
horizontal line represents the theoretical ratio of $8.3 \times 10^9$ particles/µg protein for a pure population of sEVs$^{69}$. D,E – Particle size distribution of SS-AFSC sEVs (D, n = 25) or PBS vehicle (E, n = 29) measured by NTA. Grey error bars represent SEM. F – Particle number measured in starting conditioned medium (CM), conditioned medium concentrated on Vivaspin filters (Concentrated CM) or isolated pooled SS-AFSC sEV SEC fractions 4.0 ml, 4.5 ml and 5.0 ml (SS-AFSC sEVs). Note the gradual particle loss during the process.
Figure 4-6. Characterisation of pooled sEV-rich SS-AFSC SEC fractions: sEV-specific marker content, morphology and cargo

sEVs were isolated by SEC of serum-free medium conditioned by SS-AFSCs for 48 h and sEV-rich fractions 4.0 ml, 4.5 ml and 5.0 ml were pooled and analysed. A – Levels of tetraspanin sEV-specific markers CD9, CD63 and CD81 in the SS-AFSC sEVs measured by DELFIA. n = 6. B – Dot blots of CD63 and CD81 and medium/large EV-specific marker alpha-actinin-4 (ACTN4). Note the enrichment of CD63 and CD81 in the sEVs compared to the
starting conditioned medium (Cond med) and the absence of ACTN4 in the isolated sEVs. Representative images shown: ~50 ng protein loaded per each dot. C – Transmission electron microscopy images of SS-AFSC sEVs (top panels) and SS-AFSC conditioned medium (bottom panels). Arrows point to sEVs. Scales: top left – 100 nm; top right – 500 nm; bottom left – 200 nm; bottom right – 1 μm. D,E – Functional enrichment analysis of the proteins in SS-AFSC sEVs (see section 4.3.8. for details). Top Gene Ontology (GO) Cellular Component terms are listed in panel D while panel E shows an overview of all significantly overrepresented Gene Ontology (GO) terms (Molecular Function in red, Biological Process in yellow and Cellular Component in green) in the sEV proteome. Note the top GO Cellular Component terms with very high p values in comparison to all other detected overrepresentations (circled in red). Full functional enrichment analysis can be accessed on https://drive.google.com/open?id=1fUyiEZNi_FKIXOCqjBNOBF8VN_kLDa6f.

Table 4-3. Bovine proteins detected in the SS-AFSC-conditioned medium or the SEC-isolated SS-AFSC sEVs (see the next page)

Proteins are sorted by their relative abundance in the sEV samples (peptide area by label-free quantification, LFQ). CM – conditioned medium. * enrichment of some proteins in the sEV samples is only estimated due to assignment of a minimum LFQ peptide area (3.53E+05) value for CM. $ depletion of some proteins from the sEV samples is only estimated due to assignment of a minimum LFQ peptide area (1.64E+04) value for sEVs.
<table>
<thead>
<tr>
<th>UNIPROT ID</th>
<th>Protein</th>
<th>Gene name</th>
<th>LFQ peptide area CM</th>
<th>LFQ peptide area sEVs</th>
<th>Enrichment in sEVs</th>
<th>log2 (LFQ sEVs / LFQ CM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P02453</td>
<td>Collagen alpha-1(I) chain</td>
<td>COL1A1</td>
<td>1.82E+09</td>
<td>9.84E+09</td>
<td>2.44</td>
<td></td>
</tr>
<tr>
<td>P02465</td>
<td>Collagen alpha-2(I) chain</td>
<td>COL1A2</td>
<td>1.61E+09</td>
<td>5.01E+09</td>
<td>1.64</td>
<td></td>
</tr>
<tr>
<td>P07589</td>
<td>Fibronectin</td>
<td>FN1</td>
<td>1.18E+10</td>
<td>1.12E+09</td>
<td>-3.40</td>
<td></td>
</tr>
<tr>
<td>P04258</td>
<td>Collagen alpha-1(III) chain</td>
<td>COL3A1</td>
<td>1.03E+08</td>
<td>4.10E+08</td>
<td>1.99</td>
<td></td>
</tr>
<tr>
<td>P12763</td>
<td>Alpha-2-HS-glycoprotein</td>
<td>AHSG</td>
<td>3.07E+08</td>
<td>2.58E+08</td>
<td>-0.25</td>
<td></td>
</tr>
<tr>
<td>P02769</td>
<td>Serum albumin</td>
<td>ALB</td>
<td>5.38E+08</td>
<td>5.12E+08</td>
<td>-3.39</td>
<td></td>
</tr>
<tr>
<td>Q2HJH2</td>
<td>Ras-related protein Rab-1B</td>
<td>RAB1B</td>
<td>3.53E+05</td>
<td>3.40E+07</td>
<td>6.59*</td>
<td></td>
</tr>
<tr>
<td>P49951</td>
<td>Clathrin heavy chain 1</td>
<td>CLTC</td>
<td>6.32E+06</td>
<td>2.53E+07</td>
<td>2.00</td>
<td></td>
</tr>
<tr>
<td>A2VE02</td>
<td>Junctional protein associated with coronary artery disease homolog</td>
<td>JCAD</td>
<td>3.53E+05</td>
<td>1.08E+07</td>
<td>4.94*</td>
<td></td>
</tr>
<tr>
<td>P34955</td>
<td>Alpha-1-antiproteinase</td>
<td>SERPINA1</td>
<td>1.62E+08</td>
<td>9.85E+06</td>
<td>-4.04</td>
<td></td>
</tr>
<tr>
<td>P02081</td>
<td>Hemoglobin fetal subunit beta</td>
<td>HBBF</td>
<td>1.64E+07</td>
<td>5.83E+06</td>
<td>-1.49</td>
<td></td>
</tr>
<tr>
<td>Q7SIH1</td>
<td>Alpha-2-macroglobulin</td>
<td>A2M</td>
<td>1.40E+07</td>
<td>5.46E+06</td>
<td>-1.36</td>
<td></td>
</tr>
<tr>
<td>P04896</td>
<td>Guanine nucleotide-binding protein G(s) subunit alpha isoforms short</td>
<td>GNAS</td>
<td>3.53E+05</td>
<td>4.57E+06</td>
<td>3.70*</td>
<td></td>
</tr>
<tr>
<td>Q5E9B1</td>
<td>L-lactate dehydrogenase B chain</td>
<td>LDHB</td>
<td>3.53E+07</td>
<td>3.40E+06</td>
<td>-3.37</td>
<td></td>
</tr>
<tr>
<td>P56701</td>
<td>26S proteasome non-ATPase regulatory subunit 2</td>
<td>PSMD2</td>
<td>3.53E+05</td>
<td>3.31E+06</td>
<td>3.23*</td>
<td></td>
</tr>
<tr>
<td>Q7SIB2</td>
<td>Collagen alpha-1(IV) chain</td>
<td>COL4A1</td>
<td>2.00E+07</td>
<td>2.87E+06</td>
<td>-2.80</td>
<td></td>
</tr>
<tr>
<td>Q95121</td>
<td>Pigment epithelium-derived factor</td>
<td>SERPINF1</td>
<td>3.53E+05</td>
<td>2.72E+06</td>
<td>2.95*</td>
<td></td>
</tr>
<tr>
<td>P01966</td>
<td>Hemoglobin subunit alpha</td>
<td>HBA</td>
<td>9.05E+06</td>
<td>2.62E+06</td>
<td>-1.79</td>
<td></td>
</tr>
<tr>
<td>A4FUA8</td>
<td>F-actin-capping protein subunit alpha-1</td>
<td>CAPZA1</td>
<td>3.53E+05</td>
<td>2.49E+06</td>
<td>2.82*</td>
<td></td>
</tr>
<tr>
<td>O18789</td>
<td>40S ribosomal protein S2</td>
<td>RPS2</td>
<td>3.53E+05</td>
<td>2.30E+06</td>
<td>2.70*</td>
<td></td>
</tr>
<tr>
<td>Q58DW0</td>
<td>60S ribosomal protein L4</td>
<td>RPL4</td>
<td>3.53E+05</td>
<td>1.81E+06</td>
<td>2.36*</td>
<td></td>
</tr>
<tr>
<td>Q3T133</td>
<td>Transmembrane emp24 domain-containing protein 9</td>
<td>TMED9</td>
<td>3.53E+05</td>
<td>1.64E+06</td>
<td>2.21*</td>
<td></td>
</tr>
<tr>
<td>A7YY49</td>
<td>Surfeit locus protein 4</td>
<td>SURF4</td>
<td>3.53E+05</td>
<td>1.54E+06</td>
<td>2.12*</td>
<td></td>
</tr>
<tr>
<td>P13696</td>
<td>Phosphatidylethanolamine-binding protein 1</td>
<td>PEBP1</td>
<td>1.48E+07</td>
<td>1.51E+06</td>
<td>-3.29</td>
<td></td>
</tr>
<tr>
<td>Q3T052</td>
<td>Inter-alpha-trypsin inhibitor heavy chain H4</td>
<td>ITIH4</td>
<td>3.53E+05</td>
<td>1.38E+06</td>
<td>1.97*</td>
<td></td>
</tr>
<tr>
<td>P56652</td>
<td>Inter-alpha-trypsin inhibitor heavy chain H3</td>
<td>ITIH3</td>
<td>3.53E+05</td>
<td>1.36E+06</td>
<td>1.95*</td>
<td></td>
</tr>
<tr>
<td>Q32PF2</td>
<td>ATP-citrate synthase</td>
<td>ACLY</td>
<td>1.78E+06</td>
<td>1.36E+06</td>
<td>-0.39</td>
<td></td>
</tr>
<tr>
<td>O02811</td>
<td>Phosphatidylinositol 4-kinase alpha</td>
<td>PI4KA</td>
<td>3.53E+05</td>
<td>1.20E+06</td>
<td>1.77*</td>
<td></td>
</tr>
<tr>
<td>Q58D62</td>
<td>Fetuin-B</td>
<td>FETUB</td>
<td>1.13E+07</td>
<td>1.19E+06</td>
<td>-3.25</td>
<td></td>
</tr>
<tr>
<td>Q3SZ57</td>
<td>Alpha-fetoprotein</td>
<td>AFP</td>
<td>8.82E+07</td>
<td>1.03E+06</td>
<td>-6.42</td>
<td></td>
</tr>
<tr>
<td>A2VE99</td>
<td>Septin-1</td>
<td>SEPTIN11</td>
<td>3.53E+05</td>
<td>8.60E+05</td>
<td>1.29*</td>
<td></td>
</tr>
</tbody>
</table>

*Legend on page 105*
<table>
<thead>
<tr>
<th>UNIPROT ID</th>
<th>Protein</th>
<th>Gene name</th>
<th>LFQ peptide area CM</th>
<th>LFQ peptide area sEVs</th>
<th>Enrichment in sEVs log2 [LFQ sEVs / LFQ CM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q2HJ33</td>
<td>Obg-like ATPase 1</td>
<td>OLA1</td>
<td>3.53E+05</td>
<td>8.52E+05</td>
<td>1.27*</td>
</tr>
<tr>
<td>Q3T0U2</td>
<td>60S ribosomal protein L14</td>
<td>RPL14</td>
<td>3.53E+05</td>
<td>8.26E+05</td>
<td>1.23*</td>
</tr>
<tr>
<td>Q1LZE9</td>
<td>Serine protease 23</td>
<td>PRSS23</td>
<td>1.46E+07</td>
<td>7.81E+05</td>
<td>-4.22</td>
</tr>
<tr>
<td>Q5E9V6</td>
<td>COMM domain-containing protein 4</td>
<td>COMMD4</td>
<td>3.53E+05</td>
<td>7.03E+05</td>
<td>1.00*</td>
</tr>
<tr>
<td>Q5E964</td>
<td>26S proteasome non-ATPase regulatory subunit 13</td>
<td>PSMD13</td>
<td>3.53E+05</td>
<td>6.92E+05</td>
<td>0.97*</td>
</tr>
<tr>
<td>Q3T178</td>
<td>Vacular protein sorting-associated protein 28 homolog</td>
<td>VPS28</td>
<td>3.53E+05</td>
<td>5.78E+05</td>
<td>0.71*</td>
</tr>
<tr>
<td>P41541</td>
<td>General vesicular transport factor p115</td>
<td>USO1</td>
<td>3.53E+05</td>
<td>5.23E+05</td>
<td>0.57*</td>
</tr>
<tr>
<td>P80209</td>
<td>Cathepsin D</td>
<td>CTSD</td>
<td>1.96E+07</td>
<td>5.07E+05</td>
<td>-5.27</td>
</tr>
<tr>
<td>P69678</td>
<td>Protein CutA</td>
<td>CUTA</td>
<td>3.53E+05</td>
<td>4.94E+05</td>
<td>0.49*</td>
</tr>
<tr>
<td>Q3T0D8</td>
<td>Ragulator complex protein LAMTOR1</td>
<td>LAMTOR1</td>
<td>3.53E+05</td>
<td>4.86E+05</td>
<td>0.46*</td>
</tr>
<tr>
<td>Q56JU9</td>
<td>40S ribosomal protein S24</td>
<td>RPS24</td>
<td>3.53E+05</td>
<td>4.81E+05</td>
<td>0.45*</td>
</tr>
<tr>
<td>Q3ZBD0</td>
<td>26S proteasome non-ATPase regulatory subunit 7</td>
<td>PSMD7</td>
<td>3.53E+05</td>
<td>4.48E+05</td>
<td>0.35*</td>
</tr>
<tr>
<td>Q97680</td>
<td>Thioredoxin</td>
<td>TXN</td>
<td>1.65E+07</td>
<td>1.14E+05</td>
<td>-7.18</td>
</tr>
<tr>
<td>Q3MHN5</td>
<td>Vitamin D-binding protein</td>
<td>GC</td>
<td>1.99E+07</td>
<td>1.64E+04</td>
<td>-10.25*</td>
</tr>
<tr>
<td>A2I7M9</td>
<td>Serpin A3-2</td>
<td>SERPINA3-2</td>
<td>2.17E+07</td>
<td>1.64E+04</td>
<td>-10.37*</td>
</tr>
<tr>
<td>Q9TTE1</td>
<td>Serpin A3-1</td>
<td>SERPINA3-1</td>
<td>2.32E+07</td>
<td>1.64E+04</td>
<td>-10.47*</td>
</tr>
<tr>
<td>Q29443</td>
<td>Serotransferrin</td>
<td>TF</td>
<td>2.70E+07</td>
<td>1.64E+04</td>
<td>-10.69*</td>
</tr>
<tr>
<td>Q5EA62</td>
<td>Fibulin-5</td>
<td>FBLN5</td>
<td>2.97E+07</td>
<td>1.64E+04</td>
<td>-10.82*</td>
</tr>
<tr>
<td>Q148H7</td>
<td>Keratin, type II cytoskeletal 79</td>
<td>KRT79</td>
<td>4.51E+07</td>
<td>1.64E+04</td>
<td>-11.43*</td>
</tr>
</tbody>
</table>
4.5. Discussion

In this chapter SS-AFSC sEVs were isolated and thoroughly characterised. It was demonstrated that a 48-h incubation of the SS-AFSCs in serum-free conditions leads to minor alterations in cell phenotype and viability and results in greater sEV yield and purity than shorter incubation timing. The released SS-AFSC sEVs were further systematically characterised and demonstrated to be highly pure, enriched in sEV-specific markers and devoid of non-sEV markers in comparison to the starting conditioned medium.

As suggested before for other subtypes of AFSCs\textsuperscript{154}, serum-free conditions led to a slightly reduced viability of SS-AFSCs. Both 24 h and 48 h serum-free conditions had similar cell death results and importantly the number of cells did not drop significantly from the baseline even after 48 h of serum-free culture. From the standard MSC membrane markers, a slight reduction in CD105 expression under serum-free conditions was observed, which has been reported previously for other MSCs\textsuperscript{154,288}. A morphological change involving elongation of the cells was also evident, which has been documented before\textsuperscript{288}. Importantly, previous studies demonstrated that MSC differentiation potential to mesodermal lineages is not affected despite the morphological differences and reduced CD105 marker in serum-free conditions\textsuperscript{288}.

There is only one, very recent study by Antounians et al. that uses SEC to isolate AFSC sEVs\textsuperscript{238}. The authors showed that, in comparison to precipitation and ultracentrifugation, SEC-purified sEVs are more dilute, as expected\textsuperscript{238}. They further found lower total amounts of particles in the SEC isolates. However, there are two important issues with this conclusion. Firstly, the AFSC-conditioned medium volume used by Antounians et al. is 2 ml, which is much more than the maximal volume recommended for the SEC qEVoriginal columns (iZON Science) they used (optimal loading volume 0.5 – 1 ml, see qEV brochure here: https://drive.google.com/open?id=1JuJJQn32W8NLgS0nUzhZeRepcuj76TR B, and Böing et al.\textsuperscript{160}). A delay in the elution of a part of the sEVs in the samples is expected if greater volumes are loaded so the authors could not have collected all the sEVs from a conditioned medium aliquot of this volume.
Secondly, the authors did not investigate thoroughly the sEV content of different SEC fractions and draw their conclusions about sEV yield only from 3.5 ml fraction. In this chapter it was shown that 3.5 ml fraction is likely to contain a negligible amount of the total number of sEVs and therefore the sEV numbers that can be isolated by SEC may have been grossly underestimated by Antounians et al.\textsuperscript{238}. In fact, they showed that when multiple fractions are pooled (3.5 ml – 5.5 ml), SEC isolates similar sEV numbers with comparable or even better functionality than UC- or precipitation-purified vesicles\textsuperscript{238}.

In section 3.4 it was shown that SEC co-purifies APOB\textsuperscript{+} lipoproteins with the sEVs from blood plasma. By using serum-free medium, this confounder was eliminated and the full potential of SEC for isolation of a highly-pure population of sEVs was exploited. An EV-METRIC of 67\% also placed this isolation experiment in the top 3\% of all 1,215 sEV experiments with any type of conditioned medium available in the EV-TRACK database (see EV-TRACK entry EV190058 on http://evtrack.org/review.php and section 2.3.8. for details).

A very good separation of the bulk of the soluble protein and particles was evident, while clear early peaks of particles, protein and all the assayed sEV markers coincided to suggest enrichment of sEVs with minimal soluble protein present. 48 h incubation timing yielded much higher amounts of sEVs, showed enrichment of CD9, CD63 and CD81 signal relative to total protein and had increased particle/protein ratios. Since no major phenotypic or viability differences between 24 h and 48 h were detected, 48 h incubation was chosen for subsequent sEV isolation for functional experiments (see chapters 5-7). It should be noted that incubations longer than 48 h were not investigated, and they may provide even further increases in yield and purity of the vesicles. However, careful examination of cell death should also be carried out if these are to be employed in the future, since extremely high levels of dying cells in serum-free conditions have been reported before\textsuperscript{206}. The enrichment of histones in the samples isolated here is giving an indication that such a cell death may be present. The reason for this co-isolation of histones with the sEVs is not clear but it may be attributed to the elution of DNA-nucleosomal complexes in the vesicular fraction due to their large sizes. Histone presence in the top 100 most-abundant proteins may reflect the low levels of cell death detected in serum-free conditions. It cannot be excluded, however, that there
is some form of active incorporation of histones in the sEVs although a recent study of density gradient purified sEVs suggested that sEVs are likely to be histone-free\textsuperscript{115}.

The presence of some bovine proteins in the sEV isolates was not unexpected due to the culture of the cells in serum-supplemented conditions prior to serum withdrawal. Some of the most abundant bovine proteins identified in the sEVs and the starting SS-AFSC-conditioned medium were collagens and fibronectin, which are highly similar (\(\geq 90\%\)) to their human orthologs and therefore may actually represent human proteins originating from the SS-AFSCs that were misidentified as bovine. It should be noted that the possibility that they are carried over from the prior FBS supplementation cannot be excluded. Importantly, even bovine albumin detected in the SS-AFSC-conditioned medium is mostly eliminated during the process of sEV purification as its relative abundancy is 90\% lower in the isolated sEVs compared to the starting conditioned medium. Notably, lipoproteins such as APOB were not detected in the conditioned medium or the harvested sEV samples and, as shown in chapter 3, they were by far the largest contaminants of SEC sEV isolates. Despite the fact that these sEV samples could be considered as "highly pure" with regard to published guidelines\textsuperscript{118,146} and for the preclinical research conducted here, the presence of trace bovine proteins makes them unsuitable for clinical application. Good manufacturing practice-grade methods have recently been developed for harvesting of sEVs from other cell types\textsuperscript{203} and they may also need to be established for SS-AFSC culture in order to make the sEVs clinically compatible.

As discussed in section 3.4., we have previously proposed a theoretical ratio of \(~1\times10^{10}\) particles/\(\mu\)g protein to represent a completely pure population of sEVs based solely on biochemical and biophysical parameters\textsuperscript{89}. Interestingly, an earlier study by Webber and Clayton reported particle/protein ratios as high as \(3.3\times10^{10}\) particles/\(\mu\)g protein and argued that samples with ratios of \(\geq 3\times10^{10}\) particles/\(\mu\)g protein should be considered of high purity\textsuperscript{133}. These ratios, however, have to take into account the NTA settings, operator- and time-dependent variations which may all have considerable impact\textsuperscript{155}. For instance, a Detection Threshold setting of 4 was used here for NTA measurements which was at the higher range of the one reported by Webber
and Clayton\textsuperscript{133}. Since the Detection Threshold is inversely proportional to the number of detected particles, the particle/protein ratios obtained here may have been underestimated compared to Webber and Clayton\textsuperscript{133}. Additionally, ratios of \( \geq 3 \times 10^{10} \) particles/µg protein were only obtained using CELLine Bioreactor Flasks (from Integra\textsuperscript{289}) together with sucrose cushion centrifugation isolation of sEVs, while sEVs harvested using standard 2D cell culture flasks had mean ratios of \( \approx 6 \times 10^{9} \) particles/µg protein, way below the values of \( 1.7 \times 10^{10} \) particles/µg protein obtained here\textsuperscript{133}. It would be of interest to investigate in the future whether a large-scale production of SS-AFSC sEVs using CELLine Bioreactors or similar, coupled with SEC isolation will yield particle/protein ratios higher than \( 2-3 \times 10^{10} \) particles/µg protein\textsuperscript{133}.

An interesting observation in this chapter was the loss of particles during the isolation process. Firstly, \( \sim 60\% \) of the particles in the starting conditioned medium were lost upon ultrafiltration. This may partly reflect the loss of contaminating protein aggregates and/or salt crystals during the concentration process but might also be due to loss of sEVs. Indeed, an elegant recent comparison of different ultrafiltration units demonstrated that significant proportion of the sEVs in the samples adhere to the majority of the ultrafiltration device membranes, including the Hydrosart membrane used in this report\textsuperscript{290}, despite the manufacturer’s claim for low protein binding. Thus, a selection of a different ultrafiltration membrane may be advisable for future studies to minimise these losses.

Secondly, there is a further particle loss of \( \sim 60\% \) during SEC column filtration. This also likely accounts for loss of interfering soluble protein aggregates (since \( > 95\% \) of the protein is cleared from the final sEV samples). Interestingly, however, some of the tetraspanin markers eluted after the peak sEV fractions, between 5.0 ml and 7.5 ml. Our selection of fractions 4.0, 4.5 and 5.0 ml was aimed at obtaining soluble protein-free sEVs but not at collecting the entirety of the sEVs contained within the conditioned medium. Therefore, the particle loss after SEC may predominantly be due to discarding sEV fractions contaminated with soluble protein. In support of this, similar particle recovery rates for SEC (\( \sim 40\% \)) have been previously found by others\textsuperscript{160}. 
Hence, it appears that the critical step which can be optimised to reduce the loss of sEVs is the ultrafiltration. Quantification of vesicle recoveries from conditioned medium and protein-filter adherence are beyond the scope of this report but it is advisable that future studies carefully select the filters to be used and investigate the sEV losses.

Overall, in this chapter a protocol was established to isolate highly-pure SS-AFSC sEVs using SEC and a 48-h serum-free incubation of SS-AFSCs. These culture conditions did not significantly alter SS-AFSCs morphology, viability and surface marker expression, but provided sEVs with higher yields and purity in comparison to shorter incubation timings.
Chapter 5. Cardioprotective effects of SS-AFSC sEVs in vitro and in vivo

5.1. Introduction

As discussed in section 1.6.3., MSC sEVs have previously been demonstrated to have cardioprotective activities. It has been argued that younger cells, such as foetal MSCs, and the sEVs they release may have improved potency\(^{224}\). Immature cell populations such as iPSCs\(^{216}\) or embryonic stem cell-derived MSCs\(^{65,217}\) have beneficial effects in MI models which sometimes exceed those of foetal MSCs\(^{217}\). However, there are several issues when using iPSCs or MSCs derived from embryonic progenitors. These include the potential for the formation of teratomas with certain populations of cells (e.g. iPSCs\(^{216}\)) or limited access to and ethical issues associated with some other cell types (e.g. human embryonic stem cells\(^{190,217}\)). In one study, unlike iPSCs, iPSC sEVs did not lead to cardiac tumour formation, but the follow up was only 6 weeks\(^{216}\) so it is still unclear whether sEVs from pluripotent progenitors are safe to use clinically. In fact, cell reprogramming can be induced by miRNAs alone\(^{291}\) and given the pluripotent nature of the parent cells and their propensity to form teratomas, the iPSCs sEV tumorigenicity should be more carefully examined. Such a tumour-formation effect has not been reported for foetal MSCs\(^{224}\).

AFSCs are known to exert cytoprotective effects in vitro. Previous reports showed that the secretome of AFSCs can reduce doxorubicin-induced H9c2 cardiomyoblast, neonatal rat cardiomyocytes and CPC senescence and apoptosis potentially via activation of PI3K pathway and upregulation of ABCB1B doxorubicin efflux transporter\(^{292}\). These cytoprotective and rejuvenating effects may be mediated via sEVs released by AFSCs as they were shown to reduce cell death of nitrogen mustard-treated granulosa cells\(^{235}\) and H\(_2\)O\(_2\)-treated skeletal myoblasts\(^{154}\), and to decrease H\(_2\)O\(_2\)-induced cell senescence of a lung fibroblasts cell line\(^{236}\). It should be noted, however, that the methodology of these studies only involved the use of transformed cell lines or neonatal cells along with non-physiological stress models (e.g. H\(_2\)O\(_2\)
treatment), while some investigations also used suboptimal sEV isolation protocols as discussed in section 4.1.

The AFSC secretome is also cardioprotective in vivo (see section 1.7.). In a recent study, Balbi et al. demonstrated that both AFSC-conditioned medium (non-selected for SS-AFSCs) and the ultracentrifugation-isolated sEVs have infarct size-reducing effects and improve cardiac function in a model of permanent MI in mice. Intriguingly, authors even showed that a more potent cardioprotective effect is observed with the isolated sEVs compared to the starting conditioned medium. However, no characterisation of the sEVs was presented in this study and it is not known whether the sEV samples contained any contaminants, thus precluding detailed interpretation of the results and determination of whether the sEVs are the cardioprotectant in the AFSC-conditioned medium. Furthermore, the authors used an intramyocardial administration of treatments which is unlikely to be clinically translatable in the setting of MI.

In this chapter, the effects of SS-AFSC sEVs on cytoprotection in vitro were investigated using for the first-time models of adult primary cardiomyocyte cell death, including the more physiological simulated IRI model. Additionally, the cardioprotective potential of SS-AFSC sEVs was studied in vivo using a clinically-relevant design (IRI and not permanent coronary artery ligation) together with a clinically-compatible intravenous administration and pre-reperfusion treatment. This is the first report examining the more potent SS-AFSC subtype in combination with application of a superior sEV purification method, i.e. SEC.

5.2. Aims

The aims of this chapter were to:

1) Investigate the potential of SS-AFSC sEVs to directly or indirectly (i.e. via endothelial cells) protect cardiomyocytes from IRI in vitro
2) Investigate the potential of SS-AFSC sEVs to protect the myocardium from IRI acutely, in a model of non-recovery IRI in rats in vivo
5.3. Methods

5.3.1. ROS-induced cell death

The ROS-induced cell death model is explained in detail in section 2.5.1. To generate a dose-response curve of H₂O₂-induced cell death, H₂O₂ was used at increasing concentrations (1 µM – 125 µM) in a 2-h incubation experiment.

To establish a positive control for this model, ARVCs were pretreated for 30 min with NAC, followed by addition of 40 µM of H₂O₂ for further 2 h (in the presence of NAC) (Figure 5-1).

To investigate the SS-AFSC sEV potential for protection against H₂O₂-induced ARVC death, ARVCs were pretreated for 30 min with 1 x 10¹⁰ particles/ml of SS-AFSC sEVs, followed by addition of 40 µM of H₂O₂ for further 2 h (in the presence of SS-AFSC sEVs) (Figure 5-2).

Untreated ARVCs were used as a control in all experiments.

Cell death was measured using an LDH assay (see section 2.5.3.). 25 µl of the medium or lysate were diluted to 50 µl and used in the assay. To account for differences in the starting number of cells in the H₂O₂-induced cell death assay, LDH released in the medium was normalised to the total LDH (LDH_{total} = LDH_{medium} + LDH_{lysate}).
Figure 5-1. Experimental design: Establishment of an H$_2$O$_2$-induced cell death model

Overview of the establishment of an H$_2$O$_2$-induced primary rat cardiomyocyte death model. ARVCs were treated with Vehicle (water, PBS) or increasing doses of N-acetyl-L-cysteine (NAC, 100 µM – 1 mM) for 30 min prior to the addition of H$_2$O$_2$ (white bar) and during the incubation with 40 µM H$_2$O$_2$ (black bar). LDH release was measured at the end of the incubation period and presented as a percentage of total LDH.
Figure 5-2. Experimental design: SS-AFSC sEV effects in the H₂O₂-induced cell death model

Overview of the experiment to test for effects of SS-AFSC sEVs against H₂O₂-induced death of primary rat cardiomyocytes. ARVCs were treated with Vehicle (water, PBS), N-acetyl-L-cysteine (NAC, 300 µM) or SS-AFSC sEVs (1x10^{10} sEVs/ml) for 30 min prior to the addition of H₂O₂ (white bar) and during the incubation with 40 µM H₂O₂ (black bar). LDH release was measured at the end of the incubation period and presented as a percentage of total LDH.
5.3.2. Hypoxia/reoxygenation-induced cell death (simulated IRI in vitro)

The model of cell death by simulated IRI in vitro is explained in detail in section 2.5.2.

In the first simulated IRI experiment, ARVCs were treated with varying doses of SS-AFSC sEVs \((3 \times 10^8 - 3 \times 10^9 \text{sEVs/ml})\) (Figure 5-3). Treatments started 30 min prior to hypoxia and continued throughout hypoxia and reoxygenation to achieve maximal activation (if such exists) of protective signalling cascades during both hypoxic and reoxygenation periods. To account for differences in the starting number of cells in the simulated IRI experiments, LDH release was measured in Hypoxic and Reoxygenation Buffer and normalised to the total LDH \((\text{LDH}_{\text{total}} = \text{LDH}_{\text{hypoxic}} + \text{LDH}_{\text{reoxygenation}} + \text{LDH}_{\text{lysate}})\). ARVCs incubated in normoxic conditions served as a control.

In the second simulated IRI experiment, the indirect cardioprotective potential of SS-AFSCs via effects on endothelial cells was investigated (Figure 5-4A,B). The treatments were prepared as follows:

1) For Vehicle control group, vehicle (PBS) was incubated without any cells for 3 h in Endothelial Serum-Free Defined Medium (Cell Applications Inc; 113-500, Sigma) at 37°C / 5% CO₂;

2) For sEV control group, \(1 \times 10^{10}\) SS-AFSC sEVs/ml were incubated without any cells for 3 h in Endothelial Serum-Free Defined Medium (Cell Applications Inc; 113-500, Sigma) at 37°C / 5% CO₂;

3) For HUVEC + Vehicle control group, HUVECs were incubated with vehicle (PBS) for 3 h in Endothelial Serum-Free Defined Medium (Cell Applications Inc; 113-500, Sigma) at 37°C / 5% CO₂;

4) For HUVEC + sEV group, HUVECs were incubated with \(1 \times 10^{10}\) SS-AFSC sEVs/ml for 3 h in Endothelial Serum-Free Defined Medium (Cell Applications Inc; 113-500, Sigma) at 37°C / 5% CO₂;

Two different batches of the aforementioned treatment groups were prepared and used in the experiments. For all groups, conditioned medium was collected and concentrated on Amicon Ultra-4 ultrafiltration units (3 kDa
cut-off, regenerated cellulose membranes). Final concentrate volumes were recorded, and samples were frozen at -80°C.

To mimic more closely the in vivo experiment (see sections 2.6. and 5.3.3.), ARVCs treatments were administered at reoxygenation only. Treatments were normalised by area occupied by cells and not by cell number since an accurate count of the seeded ARVCs cannot be obtained, i.e. conditioned medium from 6 area units occupied by HUVECs were added per 1 area unit occupied by ARVCs (6:1 HUVEC to ARVC area proportion). This corresponded to ~15% of the medium conditioned by a confluent layer of HUVECs in a T75 flask per each well of the 24-well plate seeded with ARVCs (Figure 5-4B).

LDH (i.e. cell death) was measured in the reoxygenation buffer only and normalised to the total LDH (LDHtotal = LDHreoxygenation + LDHlysate).

Figure 5-3. Experimental design: simulated IRI model in vitro (first experiment)
Overview of the hypoxia/reoxygenation-induced model of primary rat cardiomyocyte death (first simulated IRI experiment – see explanation in section 5.3.2. ARVCs were incubated for 5 h in hypoxic conditions (black bars) followed by 1 h reoxygenation period (white bars). Vehicle (PBS) or SS-AFSC sEVs at increasing concentrations (3x10⁸-3x10⁹ sEVs/ml) were added 30 min prior to the start of the hypoxia in culture medium (pink bars) as well as during the hypoxia and reoxygenation. Normoxic conditions were used as a control (white bar at the top). LDH release was measured at the end of the experiment and expressed as a percentage of total LDH.
Figure 5-4. Experimental design: endothelial cell-mediated protection of cardiomyocytes in the simulated IRI model *in vitro* (second experiment)

A – Schematic of the experimental design of the second simulated IRI experiment – see explanation in section 5.3.2. HUVECs were treated with SS-AFSC sEVs (1×10^{10} sEVs/ml) for 3 h. HUVEC-conditioned medium was then collected and concentrated. This conditioned medium concentrate was used in simulated IRI experiments to assay for endothelial cell-mediated cytoprotection of primary rat cardiomyocytes. B – Overview of the experimental design of the hypoxia/reoxygenation-induced model of ARVCs. Group 1 – ARVCs incubated in normoxic conditions as a control (white bar). Group 2 and 3 – ARVCs were incubated for 5 h in hypoxic conditions (black bars) followed by 1 h reoxygenation period (white bars). Treatments were neat medium containing either vehicle (PBS, Group 2) or SS-AFSC sEVs (1×10^{10} sEVs/ml, Group 3). Group 4 and 5 – ARVCs were incubated for 5 h in hypoxic conditions (black bars) followed by 1 h reoxygenation period (white bars). Treatments were medium conditioned by HUVECs treated with either vehicle (PBS, Group 4) or SS-AFSC sEVs (1×10^{10} sEVs/ml, Group 5). LDH release was measured at the end of the experiment and expressed as a percentage of total LDH.
5.3.3. *In vivo model of non-recovery IRI in rats*

*In vivo* experiments were performed as described in section 2.6. Rats received 500 µl/animal (single bolus intravenous dose) containing vehicle (PBS), bradykinin (positive control for cardioprotection, 40 µg/kg; B3259, Sigma) or SS-AFSC sEVs (2x10^{11} particles/animal; ~1x10^{10} particles/ml blood) (Figure 5-5). Two different batches of SS-AFSC sEVs were prepared and used in the experiments and the doses of protein administered were 42-72 µg/kg.

5.3.4. Proteomics

Proteomic analysis is performed as per section 4.3.8.
Figure 5-5. Experimental design: non-recovery IRI in rats in vivo

Overview of the non-recovery myocardial IRI model in rats. LAD artery was occluded for 30 min (black bar) followed by reperfusion for 2 h (white bar). Treatments were administered intravenously (i.v.) 2 min prior to reperfusion and included vehicle (PBS, top panel), bradykinin (40 µg/kg, positive control, middle panel) and SS-AFSC sEVs (2x10^{11} sEVs/animal, bottom panel). At the end of reperfusion, hearts were collected, stained and processed as explained in sections 2.6.3. and 2.6.4.
5.4. Results

5.4.1. Cardioprotective effects of SS-AFSC sEVs in vitro: direct protection of isolated cardiomyocytes

In this section the cardioprotective potential of SS-AFSC sEVs was investigated. Initial experiments used primary adult cardiomyocytes in vitro, terminally-differentiated cells that have not previously been employed in studies with any MSC EVs. Secondly, an in vivo rat model of MI and a clinically-compatible administration treatment were employed which, if successful, would be easily translatable to clinic. Finally, a model was used for studying whether SS-AFSC sEVs can induce secretion of cardioprotective factors from primary endothelial cells which in turn protect ARVCs in vitro.

Since ROS can induce cell death during IRI\textsuperscript{24} and ROS reduction is protective in this setting\textsuperscript{55}, a model of hydrogen peroxide (H\textsubscript{2}O\textsubscript{2})-induced death of ARVCs was used. The model was established by generating a dose-response curve of H\textsubscript{2}O\textsubscript{2}-induced ARVC death. LDH release from ARVCs, as a surrogate measurement of cell death, increased dose-dependently with an EC\textsubscript{50} of 41 ± 4 µM (Figure 5-6A). Therefore, the 40 µM dose was selected for further experiments.

In a dose-response experiment, N-acetyl-L-cysteine (NAC) was shown to effectively protect ARVCs from H\textsubscript{2}O\textsubscript{2}-induced cell death as it markedly reduced LDH release at all concentrations (44 ± 13% for H\textsubscript{2}O\textsubscript{2} + Vehicle vs 17 ± 5% for 100 µM NAC (p < 0.05), 9 ± 1% for 300 µM NAC (p < 0.01) and 11 ± 1% for 1 mM NAC (p < 0.01); Figure 5-6B,C). The most consistent protection was conferred by 300 µM NAC which was the dose selected for use as positive control in the subsequent experiment.

In a dose-response experiment, N-acetyl-L-cysteine (NAC) was shown to effectively protect ARVCs from H\textsubscript{2}O\textsubscript{2}-induced cell death as it markedly reduced LDH release at all concentrations (44 ± 13% for H\textsubscript{2}O\textsubscript{2} + Vehicle vs 17 ± 5% for 100 µM NAC (p < 0.05), 9 ± 1% for 300 µM NAC (p < 0.01) and 11 ± 1% for 1 mM NAC (p < 0.01); Figure 5-6B,C). The most consistent protection was conferred by 300 µM NAC which was the dose selected for use as positive control in the subsequent experiment.

Next, the protective potential of SS-AFSC sEVs against H\textsubscript{2}O\textsubscript{2}-induced ARVC death was investigated. As expected, the positive control NAC used at 300 µM reduced LDH release from 23 ± 4% (H\textsubscript{2}O\textsubscript{2} + Vehicle group) to 8 ± 1% (300 µM NAC group, p < 0.01; Figure 5-7A,B). However, no protective effect of SS-AFSC sEVs was observed in this model (31 ± 4%, p > 0.05 vs H\textsubscript{2}O\textsubscript{2} + Vehicle) (Figure 5-7A,B).
To assess the cardioprotective activity of SS-AFSC sEVs in a more physiological model, ARVCs were subjected to simulated IRI involving 5 hours of hypoxia followed by 1 hour of reoxygenation *in vitro* with pre-, per- and post-hypoxia treatment with vesicles (Figure 5-3). The LDH release of ARVCs in control condition (*i.e.* normoxia) was 8 ± 2% which was increased significantly to 23 ± 2% after hypoxia/reoxygenation (*p* < 0.001; Figure 5-8A,B). Nevertheless, none of the doses of SS-AFSC sEVs had protective effects in this model (*p* > 0.05; 22 ± 3% for 3x10⁸ particles/ml SS-AFSC sEVs, 22 ± 3% for 1x10⁹ particles/ml SS-AFSC sEVs; Figure 5-8A,B). Despite the lack of protection, proteomic analysis of the sEV samples showed significant enrichment of proteins associated with the Gene Ontology Biological Process term “negative regulation of cell death” (53 proteins of this category are present in the SS-AFSC sEV samples with 1.9 times enrichment compared to the randomly expected number, *p* = 0.01, Table 5-1). Some of the proteins listed in Table 5-1, such as heat shock proteins²⁹³, annexins²⁹⁴,²⁹⁵, caveolins²⁹⁶ etc., have previously been shown to possess cardioprotective properties, while others which are not in this list can also be protective against IRI (*e.g.* PTX³²⁹⁷, see discussion in section 5.5).

Overall, no cytoprotective effects of SS-AFSC sEVs on isolated ARVCs *in vitro* were observed. Yet, in an intact whole organism the effects may be different to what is observed *in vitro*. Furthermore, a number of proteins in the sEV isolates may have cardioprotective effects as identified by proteomic analysis. Hence, an *in vivo* model of IRI was employed next.
Figure 5-6. Establishment of a model of H₂O₂-induced ARVC death

A – ARVCs were incubated with increasing concentrations of H₂O₂ (0-125 µM) for 2 h. LDH release was measured at the end of the incubation period and presented as a percentage of total LDH. H₂O₂ dose-dependently increased ARVC death with an EC₅₀ = 41 ± 4 µM (r² = 0.8667), n = 6. B – ARVCs were treated with Vehicle (water) or increasing concentrations of N-acetylcysteine (NAC, 100-1000 µM) 30 min before addition of H₂O₂ and during the H₂O₂ incubation ([H₂O₂] = 40 µM). *** p < 0.001, * p < 0.05, ** p < 0.01, 1-way Repeated Measures ANOVA with Dunnett’s post-hoc test. LDH release calculated as per panel A. n = 4. C – Representative images of B. Scale: 200 µm.
Figure 5-7. Effects of SS-AFSC sEVs on H$_2$O$_2$-induced ARVC death

A – ARVCs were treated with Vehicle (water, PBS), N-acetylcysteine (NAC, 300 µM) or SS-AFSC sEVs (1x10$^{10}$ sEVs/ml) 30 min before addition of H$_2$O$_2$ and during the H$_2$O$_2$ incubation ([H$_2$O$_2$] = 40 µM). LDH release was measured at the end of the incubation period and presented as a percentage of total LDH. ** p < 0.01, 1-way Repeated Measures ANOVA with Dunnett's post-hoc test. n = 3. B – Representative images of A. Scale: 200 µm.
Figure 5-8. Effects of SS-AFSC sEVs on simulated IRI in vitro
A – ARVCs were incubated for 5 h in hypoxic conditions followed by 1 h reoxygenation period. SS-AFSC sEVs at increasing concentrations (3x10^8-3x10^9 sEVs/ml) were added 30 min prior to the start of the hypoxia as well as during the hypoxia and reoxygenation. Normoxic conditions were used as a control. LDH release was measured at the end of the experiment and expressed as a percentage of total LDH. H/R – hypoxia/reoxygenation. *** p < 0.001, 1-way Repeated Measures ANOVA with Dunnett’s post-hoc test. n = 7. B – Representative images of A. Scale: 200 µM.

Table 5-1. Proteins associated with the Gene Ontology Biological Process term “negative regulation of cell death” in SS-AFSC sEV samples (see the next page)
SS-AFSC sEV proteomic analysis was performed as explained in sections 2.3.6. and 4.3.8. The table below shows proteins detected in the SS-AFSC sEV sample which are associated with the Gene Ontology Biological Process term “negative regulation of cell death” ordered by their relative abundancy by LFQ peptide area. LFQ – label-free quantification. PSM – peptide-spectrum match.
<table>
<thead>
<tr>
<th>UNIPROT ID</th>
<th>Protein</th>
<th>Gene name</th>
<th>Peptide area (LFQ)</th>
<th>PSM count</th>
</tr>
</thead>
<tbody>
<tr>
<td>P02458</td>
<td>Collagen alpha-1(I) chain</td>
<td>COL2A1</td>
<td>1.52E+09</td>
<td>136</td>
</tr>
<tr>
<td>P68032</td>
<td>Actin, alpha cardiac muscle 1</td>
<td>ACTC1</td>
<td>9.77E+08</td>
<td>291</td>
</tr>
<tr>
<td>P08758</td>
<td>Annexin A5</td>
<td>ANXA5</td>
<td>4.53E+08</td>
<td>252</td>
</tr>
<tr>
<td>P0CG48</td>
<td>Polyubiquitin-C</td>
<td>UBC</td>
<td>2.90E+08</td>
<td>81</td>
</tr>
<tr>
<td>P04083</td>
<td>Annexin A1</td>
<td>ANXA1</td>
<td>2.26E+08</td>
<td>83</td>
</tr>
<tr>
<td>P05556</td>
<td>Integrin beta-1</td>
<td>ITGB1</td>
<td>1.67E+08</td>
<td>145</td>
</tr>
<tr>
<td>P23528</td>
<td>Coffin-1</td>
<td>CFL1</td>
<td>6.89E+07</td>
<td>44</td>
</tr>
<tr>
<td>P02768</td>
<td>Serum albumin</td>
<td>ALB</td>
<td>6.56E+07</td>
<td>37</td>
</tr>
<tr>
<td>Q15672</td>
<td>Twist-related protein 1</td>
<td>TWIST1</td>
<td>4.14E+07</td>
<td>4</td>
</tr>
<tr>
<td>P04792</td>
<td>Heat shock protein beta-1</td>
<td>HSPB1</td>
<td>1.60E+07</td>
<td>35</td>
</tr>
<tr>
<td>P06756</td>
<td>Integrin alpha-V</td>
<td>ITGAV</td>
<td>1.56E+07</td>
<td>68</td>
</tr>
<tr>
<td>O14786</td>
<td>Neurophilin-1</td>
<td>NRP1</td>
<td>1.56E+07</td>
<td>26</td>
</tr>
<tr>
<td>P06733</td>
<td>Alpha-ensolase</td>
<td>ENO1</td>
<td>1.28E+07</td>
<td>77</td>
</tr>
<tr>
<td>P04899</td>
<td>Guanine nucleotide-binding protein G(i) subunit alpha-2</td>
<td>GNAI2</td>
<td>1.04E+07</td>
<td>58</td>
</tr>
<tr>
<td>P61586</td>
<td>Transforming protein RhoA</td>
<td>RHOA</td>
<td>1.03E+07</td>
<td>25</td>
</tr>
<tr>
<td>Q03135</td>
<td>Cavelolin-1</td>
<td>CAV1</td>
<td>9.06E+06</td>
<td>12</td>
</tr>
<tr>
<td>P11021</td>
<td>Endoplasmic reticulum chaperone BiP</td>
<td>HSPA5</td>
<td>8.75E+06</td>
<td>31</td>
</tr>
<tr>
<td>P18085</td>
<td>ARB-ribosylation factor 4</td>
<td>ARF4</td>
<td>7.05E+06</td>
<td>36</td>
</tr>
<tr>
<td>P08754</td>
<td>Guanine nucleotide-binding protein G(i) subunit alpha</td>
<td>GNAI3</td>
<td>6.53E+06</td>
<td>34</td>
</tr>
<tr>
<td>P35222</td>
<td>Catenin beta-1</td>
<td>CTNNB1</td>
<td>6.52E+06</td>
<td>37</td>
</tr>
<tr>
<td>Q07954</td>
<td>Prolow-density lipoprotein receptor-related protein 1</td>
<td>LRP1</td>
<td>6.04E+06</td>
<td>42</td>
</tr>
<tr>
<td>P22392</td>
<td>Nucleoside diphosphate kinase B</td>
<td>NME2</td>
<td>5.94E+06</td>
<td>6</td>
</tr>
<tr>
<td>P35221</td>
<td>Catenin alpha-1</td>
<td>CTNNA1</td>
<td>5.50E+06</td>
<td>28</td>
</tr>
<tr>
<td>P07996</td>
<td>Thrombospondin-1</td>
<td>THBS1</td>
<td>5.30E+06</td>
<td>45</td>
</tr>
<tr>
<td>P08238</td>
<td>Heat shock protein HSP 90-beta</td>
<td>HSP90B1</td>
<td>5.01E+06</td>
<td>13</td>
</tr>
<tr>
<td>P21333</td>
<td>Filamin-A</td>
<td>FLNA</td>
<td>5.01E+06</td>
<td>91</td>
</tr>
<tr>
<td>P36955</td>
<td>Pigment epithelium-derived factor</td>
<td>SERPINF1</td>
<td>4.73E+06</td>
<td>39</td>
</tr>
<tr>
<td>P63104</td>
<td>14-3-3 protein zeta/delta</td>
<td>YWHAZ</td>
<td>4.49E+06</td>
<td>25</td>
</tr>
<tr>
<td>P09525</td>
<td>Annexin A4</td>
<td>ANXA4</td>
<td>3.99E+06</td>
<td>20</td>
</tr>
<tr>
<td>P09211</td>
<td>Glutathione S-transferase P</td>
<td>GSTP1</td>
<td>3.47E+06</td>
<td>14</td>
</tr>
<tr>
<td>P16070</td>
<td>CD44 antigen</td>
<td>CD44</td>
<td>2.97E+06</td>
<td>18</td>
</tr>
<tr>
<td>P01112</td>
<td>GTPase HRas</td>
<td>HRAS</td>
<td>2.85E+06</td>
<td>15</td>
</tr>
<tr>
<td>P0DMV8</td>
<td>Heat shock 70 kDa protein 1A</td>
<td>HSPA1A</td>
<td>2.80E+06</td>
<td>16</td>
</tr>
<tr>
<td>O95865</td>
<td>N(G),N(G)-dimethylarginine dimethylaminohydrolase 2</td>
<td>DDH2</td>
<td>2.74E+06</td>
<td>8</td>
</tr>
<tr>
<td>P62255</td>
<td>14-3-3 protein epsilon</td>
<td>YWHAE</td>
<td>2.06E+06</td>
<td>10</td>
</tr>
<tr>
<td>P32119</td>
<td>Peroxiredoxin-2</td>
<td>PRDX2</td>
<td>2.01E+06</td>
<td>6</td>
</tr>
<tr>
<td>P52565</td>
<td>Rho GDP-dissociation inhibitor 1</td>
<td>ARHGDI</td>
<td>1.99E+06</td>
<td>2</td>
</tr>
<tr>
<td>P09619</td>
<td>Platelet-derived growth factor receptor beta</td>
<td>PDGFBR</td>
<td>1.77E+06</td>
<td>6</td>
</tr>
<tr>
<td>P41221</td>
<td>Protein Wnt-5a</td>
<td>WNT5A</td>
<td>1.64E+06</td>
<td>10</td>
</tr>
<tr>
<td>P02545</td>
<td>Prelamin-A/C</td>
<td>LMNA</td>
<td>1.53E+06</td>
<td>6</td>
</tr>
<tr>
<td>P06744</td>
<td>Glucose-6-phosphate isomerase</td>
<td>GPI</td>
<td>1.51E+06</td>
<td>5</td>
</tr>
<tr>
<td>P14625</td>
<td>Endoplasmin</td>
<td>HSP90B1</td>
<td>1.30E+06</td>
<td>8</td>
</tr>
<tr>
<td>O14558</td>
<td>Heat shock protein beta-6</td>
<td>HSPB6</td>
<td>1.28E+06</td>
<td>9</td>
</tr>
<tr>
<td>P08648</td>
<td>Integrin alpha-5</td>
<td>ITGA5</td>
<td>1.17E+06</td>
<td>6</td>
</tr>
<tr>
<td>Q96TA1</td>
<td>Niban-like protein 1</td>
<td>FAM129B</td>
<td>1.06E+06</td>
<td>7</td>
</tr>
<tr>
<td>P02511</td>
<td>Alpha-crystallin B chain</td>
<td>CRYAB</td>
<td>1.04E+06</td>
<td>2</td>
</tr>
<tr>
<td>Q04721</td>
<td>Neurogenic locus notch homolog protein 2</td>
<td>NOTCH2</td>
<td>8.68E+05</td>
<td>4</td>
</tr>
<tr>
<td>Q9Y4L1</td>
<td>Hypoxia up-regulated protein 1</td>
<td>HYOU1</td>
<td>7.80E+05</td>
<td>4</td>
</tr>
<tr>
<td>P17931</td>
<td>Galectin-3</td>
<td>LGALS3</td>
<td>7.37E+05</td>
<td>4</td>
</tr>
<tr>
<td>P63244</td>
<td>Receptor of activated protein C kinase 1</td>
<td>RACK1</td>
<td>6.81E+05</td>
<td>2</td>
</tr>
<tr>
<td>Q9H444</td>
<td>Charged multivesicular body protein 4b</td>
<td>CHMP4B</td>
<td>5.81E+05</td>
<td>2</td>
</tr>
<tr>
<td>P61978</td>
<td>Heterogeneous nuclear ribonucleoprotein K</td>
<td>HNRNPK</td>
<td>3.91E+05</td>
<td>2</td>
</tr>
<tr>
<td>P61247</td>
<td>40S ribosomal protein S3a</td>
<td>RPS3A</td>
<td>3.66E+05</td>
<td>2</td>
</tr>
</tbody>
</table>
### 5.4.2. Cardioprotective effects of SS-AFSC sEVs *in vivo*

To test the hypothesis that SS-AFSC sEVs can protect the myocardium *in vivo*, a rat model of IRI was used. Rats were subjected to 30 min myocardial ischaemia, induced by LAD coronary artery ligation, followed by 2 h reperfusion (Figures 2-3 and 2-4). Vehicle, bradykinin (positive control) or SS-AFSC sEVs (2x10^{11} particles/animal; ~1x10^{10} particles/ml blood) were injected intravenously, 2 minutes prior to reperfusion to mimic a clinically-applicable administration of treatments (Figure 5-5). As expected, bradykinin reduced infarct size (IS) from 71 ± 5% (Vehicle group) to 47 ± 6% (Bradykinin group) (p < 0.05, Figure 5-9A,C). Intriguingly, SS-AFSC sEVs also had cardioprotective effects decreasing the size of the infarcts to 44 ± 7% which represented a relative reduction of 38% compared to the Vehicle group (p < 0.05, Figure 5-9A,C). The ischaemic area (i.e. area at risk, AAR) was not different between the groups (Vehicle: 46 ± 3%, Bradykinin: 37 ± 2%, SS-AFSC sEVs: 37 ± 4; p > 0.05, Figure 5-9B) and the weights of the rats were also similar (p > 0.05, Figure 5-10A).

This experiment was performed in a randomised, blinded manner, involving two different investigators in measurements of IS and AAR. Both IS and AAR estimations correlated very well between the investigators (p < 0.0001 for both IS and AAR, Figure 5-10B) while the discrepancies between measurements recorded by the two investigators were within the acceptable range and did not differ significantly between the treatment groups (p > 0.05 for both IS and AAR, Figure 5-10C).

Taken together, this indicates that SS-AFSC sEVs are potent cardioprotective agents against IRI *in vivo*, despite the lack of direct effects on cardiomyocytes in simulated IRI *in vitro* (see section 5.4.1). Thus, an indirect effect of myocardial salvaging is likely to exist, *e.g.* via effect of SS-AFSC sEVs on endothelial cells or other body organs or systems.
Figure 5-9. Effects of SS-AFSC sEVs on IRI in rats in vivo

Rats underwent a 30-min myocardial ischaemia, induced by LAD artery ligation, followed by reperfusion for 2 h. Treatments were administered intravenously, 2 min prior to reperfusion. Treatments included vehicle (PBS), bradykinin (40 µg/kg) and SS-AFSC sEVs (2x10¹¹ sEVs/rat). A – Infarct size as a percentage of the area at risk (ischaemic area). n = 6. * p < 0.05, 1-way ANOVA with Dunnett’s post-hoc test. B – Area at risk as a percentage of left ventricle area. n = 6. ns – non-significant (p > 0.05), 1-way ANOVA. C – Representative images of hearts stained with Evans Blue and TTC for all groups. White areas represent infarcted tissue.
Figure 5-10. IRI \textit{in vivo}: animal weights and variability of the analyses

A – Weights of the rats used for IRI \textit{in vivo}. p > 0.05, 1-way ANOVA. B,C – variability of the infarct size and area at risk analysed by two blinded investigators. B – Correlation of infarct size (IS as a percentage of AAR, left panel) and area at risk (AAR as a percentage of left ventricle area, right panel) measurements of the two investigators. IS, left panel: p < 0.0001; Pearson’s correlation test, Pearson \( r = 0.952 \). AAR, right panel: p < 0.0001; Pearson’s correlation test, Pearson \( r = 0.942 \). C – Discrepancies in infarct size (left panel) and area at risk (right panel) measurements of the two investigators. ns – non-significant (p > 0.05), 1-way ANOVA.
5.4.3. Cardioprotective effects of SS-AFSC sEVs *in vitro*: indirect protection of isolated cardiomyocytes by effects on endothelial cells

To investigate further the cardioprotection observed *in vivo*, a model to assess the potential of SS-AFSC sEVs to indirectly deliver these benefits via effects on endothelial cells was used. Firstly, conditioned medium from HUVECs treated with SS-AFSC sEVs (1x10^{10} sEVs/ml) or vehicle was collected and concentrated as explained in section 5.3.2. ARVCs were then subjected to simulated IRI *in vitro* and treated with HUVEC-conditioned medium. Treatments were given at reoxygenation to mimic the *in vivo* administration of SS-AFSC sEVs just prior to reperfusion. As expected, hypoxia/reoxygenation increased cell death significantly compared to normoxic incubation (10 ± 1% for Normoxia vs 31 ± 3% for Vehicle, p < 0.001, Figure 5-11). Similar to the observations in section 5.4.1, the presence of SS-AFSC sEVs alone at a dose similar to the one achieved *in vivo* in section 5.4.2. did not protect ARVCs in simulated IRI (31 ± 3% for Vehicle vs 31 ± 2% for sEVs, p > 0.05, Figure 5-11). Interestingly, non-treated HUVEC-conditioned medium and sEV-treated HUVEC-conditioned medium were also found not to be protective against hypoxia/reoxygenation injury of ARVCs (31 ± 3% for Vehicle vs 31 ± 3% for non-treated HUVECs and 31 ± 4% for sEV-treated HUVECs, p > 0.05 for both, Figure 5-11). This indicates that the stable secretome of sEV-treated endothelial cells is not mediating the cardioprotective effects of SS-AFSC sEVs.

Overall, SS-AFSC sEVs protected rat hearts from IRI *in vivo*, indicating that foetal sEVs may be a promising novel cardioprotective therapeutic. Intriguingly, this did not seem to be mediated by direct effects on cardiomyocytes or by indirect effects via inducing a release of a stable cardioprotective agent from endothelial cells as shown by *in vitro* experiments. Therefore, the mechanisms behind the powerful cardioprotective effects of SS-AFSC sEVs *in vivo* remain open for further exploration.
Figure 5-11. Effects of the stable sEV-treated HUVEC secretome on simulated IRI in vitro

ARVCs were incubated for 5 h in hypoxic conditions followed by 1 h reoxygenation period. ARVCs were treated with non-conditioned (Vehicle or sEVs at 1x10^10 sEVs/ml) or HUVEC-conditioned medium (vehicle-treated: HUVEC + Vehicle, or SS-AFSC sEV-treated: HUVEC + sEVs at 1x10^10 sEVs/ml) during reoxygenation only (see section 5.3.2. for details). Normoxic conditions were used as a control. LDH release was measured at the end of the experiment and expressed as a percentage of total LDH. H/R – hypoxia/reoxygenation. *** p < 0.001, ns – non-significant (p > 0.05), 1-way Repeated Measures ANOVA with Tukey’s post-hoc test. n = 5.
5.5. Discussion

In this chapter, the activity of SS-AFSC sEVs on models of IRI in vitro and in vivo was investigated. Using a clinically-compatible model of acute IRI in rats, it was shown for the first time that SS-AFSC sEVs are cardioprotective if administered intravenously prior to reperfusion. Intriguingly, no pro-survival effects of SS-AFSC sEVs on simulated IRI of primary adult cardiomyocytes were observed in vitro, suggestive of indirect cardioprotective functions. To investigate a potential endothelial-mediated cardioprotection, the effects of medium conditioned by SS-AFSC sEVs-treated endothelial cells on simulated IRI were examined. No cytoprotective effects were seen in this model either, indicating that the indirect cardioprotective effects are mediated by non-stable factors released by endothelial cells or by other cells/organs of the body.

Using the improved and systematically characterised sEV isolation methodology presented in chapter 4, it was demonstrated for the first time that SS-AFSC sEVs can protect the animal heart from IRI when using a clinically-applicable administration technique and injection timing, i.e. intravenous injection just prior to myocardial reperfusion. While previous findings indicated that sEVs isolated from adult multipotent progenitors may have benefits in myocardial infarction setting, their translational potential is unclear because of ethical concerns (e.g. using embryonic stem cells to derive MSCs) or difficulty of the process for obtaining the cells (e.g. differentiation of embryonic stem cells or cardiac biopsies from diseased patients). Our finding that SS-AFSC sEVs are cardioprotective when injected intravenously is especially important in light of a series of recent reports demonstrating that sEVs of various origins cannot exert cardioprotective benefits if delivered to the bloodstream. For example, sEVs derived from CDCs reduce infarct size and improve heart function in pigs subjected to IRI only when administered intramyocardially and not intracoronary. Similarly, intravenous injection of MSC sEVs was not effective in improving myocardial blood flow and heart function in a model of permanent coronary artery ligation in pigs, while intramyocardial delivery improved cardiac output and stroke volume, and increased vessel numbers in ischaemic tissue via activation of ERK, AKT and eNOS. Since intravenous administration of MSCs or MSC-conditioned
medium\textsuperscript{189} is cardioprotective, it could be speculated that the intravenous cardioprotection of SS-AFSC sEVs observed here may be due to their more potent foetal nature. In support of this, the dose of sEVs administered here with regard to their protein content was $\sim40$-$70$ $\mu$g/kg. Curiously, other studies investigating the effects of foetal stem cell-derived sEV types in a MI setting report much higher doses required for effective protection of the heart. These include foetal limb MSCs used at $\sim330$-$400$ $\mu$g/kg in a model of mice MI\textsuperscript{217}, umbilical cord MSCs at $\sim1600$-$1800$ $\mu$g/kg in a model of rat MI\textsuperscript{191}, and even AFSCs that were not selected for the potent SS-AFSC population at $\sim150$-$180$ $\mu$g/kg in a model of mice MI\textsuperscript{233}. A higher dose of 300 $\mu$g/kg CPC sEVs was also necessary for improvement of heart function in a rat model of MI as a lower dose of 30 $\mu$g/kg was ineffective\textsuperscript{196}. It is important to note that there is one study where authors used a lower dose than the one used here. Arslan et al. documented efficient cardioprotection \textit{in vivo} with a lower dose limit of 4 $\mu$g/kg of sEVs harvested from human embryonic stem cell-derived MSCs\textsuperscript{190}, leading some to claim that embryonic stem cell-derived MSC sEVs are particularly potent\textsuperscript{217}. However, human embryonic stem cells are not easily accessible and there are ethical issues associated with their research use. In addition, at the time of publication of this article, guidelines for sEV isolation and characterisation by the International Society of Extracellular Vesicles\textsuperscript{110,118} were not yet in place. Hence, the authors did not analyse their isolates for soluble protein contaminants and therefore it remains unclear whether the observed effects and the high cardioprotective potency of the samples are only mediated via the sEVs or also via non-vesicular contaminants by achieving multitarget effects. Finally, no experiments with doses of $<40$ $\mu$g/kg of vesicles were performed here, and it remains to be seen whether SS-AFSC sEVs can protect the heart at doses of $\leq4$ $\mu$g/kg.

In the setting of IRI, ROS generation during reperfusion is believed to contribute to reperfusion injury\textsuperscript{24} and therefore protection against ROS-induced cell death can mitigate IRI\textsuperscript{55}. Previous reports showed that EVs can carry antioxidant enzymes such as superoxide dismutase-1\textsuperscript{165} and if loaded with exogenous enzymes involved in antioxidant response, vesicles can successfully deliver them to recipient cells\textsuperscript{303}. Furthermore, MSC sEVs exhibited antioxidant properties in chemotherapy-induced ROS models \textit{in vitro}
and in vivo\textsuperscript{304} and normalised glutathione and superoxide dismutase levels in colitis\textsuperscript{305} and sepsis-induced lung injury\textsuperscript{306} models. Here, a model of H\textsubscript{2}O\textsubscript{2}-induced ARVC death was successfully established and showed that SS-AFSC sEVs provide no acute cytoprotection against ROS-induced cell death. Conversely, one other study by Balbi et al. used an H\textsubscript{2}O\textsubscript{2}-induced cell stress model in vitro and found protective effects of sEVs derived from a population of AFSCs (not selected for SS-AFSC)\textsuperscript{154}. It should be noted however, that authors used different sEV isolation protocol (i.e. UC), considerably higher H\textsubscript{2}O\textsubscript{2} concentrations and a skeletal myoblast cell line\textsuperscript{154}. Importantly, Balbi et al. stressed the cells with H\textsubscript{2}O\textsubscript{2} for 2 h but investigated longer-term effects after a 24-h washout period\textsuperscript{154} while in this thesis, the effects of SS-AFSC sEVs were tested in an acute model. Therefore, major differences are present between the past report and the current study, and it cannot be excluded that SS-AFSC sEVs may be protective in the model used by Balbi et al. or in another cell type.

In this study the effects of SS-AFSC sEVs were investigated in a physiological hypoxia/reoxygenation-induced primary cardiomyocyte death model. Intriguingly, SS-AFSC sEVs were not prosurvival despite their potential to reduce infarct size in the rat IRI model. In vivo cardioprotection may also be mediated via an indirect mechanism, e.g. through effects on other organs of the body\textsuperscript{173} or on the endothelial cells\textsuperscript{299,300} which may subsequently release pro-survival factors acting as paracrine or endocrine cardioprotectants. To address this, endothelial cells were treated with SS-AFSC sEVs and conditioned medium was collected and administered in the simulated IRI model. However, no protection was observed in this model as well. It is unlikely that this observation is due to a dosing issue since the sEV concentration used here is expected to be at least equal to the one achieved in vivo (i.e. 1\times10\textsuperscript{10} sEVs/ml) and in fact may even be higher because of in vivo sEV elimination or spleen and liver trapping\textsuperscript{307}. Furthermore, these data do not completely exclude the possibility that endothelial cells mediate cardioprotection by SS-AFSC sEVs due to several limitations of the current approach. Firstly, HUVECs were used here because of their primary nature, easy accessibility and well-described characteristics. However, they have foetal nature and it may be advisable to use a cell population with cardiac origin in the future studies, e.g.
primary cardiac left ventricular microvascular endothelial cells\textsuperscript{308}, which may be more suited for experiments studying endothelial-mediated cardioprotection. Secondly, the model described here can only assay stable cardioprotectant molecules released by the endothelial cells, since sEV-stimulated HUVEC-conditioned medium was harvested, concentrated on ultrafiltration units and frozen. Moreover, it is expected that small factors, below the ultrafiltration device membrane cut-off of 3 kDa, will be lost during the concentration step. For instance, one promising candidate may be nitric oxide (NO) – a cardioprotective gas\textsuperscript{309} with molecular weight of only 30 Da and a half-life in biological systems of just a few seconds\textsuperscript{310}. In fact, SDF1 and EDN1 are two known cardioprotective proteins\textsuperscript{311–313} detected in the SS-AFSC sEV samples by protein arrays (see section 7.1.). SDF1 induces activation of endothelial nitric oxide synthase (eNOS) in bovine aortic endothelial cells\textsuperscript{314}, while EDN1 can act on ETB receptors on bovine carotid artery endothelial cells to increase production of NO\textsuperscript{309}. Furthermore, two further NO regulators – RTN4 and BGN, are very abundant proteins in SS-AFSC sEVs as shown by the proteomic analysis (see section 7.1. and http://drive.google.com/open?id=1fUyiEZNi_iFKIXOCqjBNOBF8VN_kLDa6f). RTN4B isoform may be responsible for induction of NO release through actions on its receptor NgBR since overexpression of NgBR led to eNOS phosphorylation and activation in pulmonary aortic endothelial cells and its knockdown caused opposing effects\textsuperscript{315}. BGN, on the other hand, reduced cell death in a model of hypoxia/reoxygenation of neonatal cardiomyocytes by induction of eNOS and NO release\textsuperscript{316}. Whether BGN also induces NO release in endothelial cells remains unknown. Notably, there are also proteins identified in the SS-AFSC sEV samples with capabilities of reducing NO release, such as PTX3\textsuperscript{317}, and the balance between stimulators and inhibitors of NO production will be important for the overall biological effect of the sEVs which should be tested empirically.

Finally, there is a possibility that eNOS is carried by the SS-AFSC sEVs. Despite the fact that eNOS was not detectable by proteomic analysis here, it cannot be excluded that it was present in the vesicular cargo at levels below the limit of detection for the LC-MS/MS technique. In support of this, it has previously been shown that eNOS is carried in the vesicles of foetal origin (i.e.
placental sEVs)\textsuperscript{318} and it will therefore be intriguing to investigate the possibility that foetal SS-AFSC sEVs carry this enzyme, \textit{e.g.} by using more targeted techniques such as immunoassays or immunoblotting.

It is also plausible that SS-AFSC sEV protective activity is mediated via effects on other organs or cells, and not the endothelium. For instance, it has been proposed that CDC sEVs are cardioprotective via macrophage polarisation\textsuperscript{67} (see also section 1.6.3.) and Pickard \textit{et al.} showed that nervous system engagement is crucial for myocardial salvage by soluble factors released after application of RIC\textsuperscript{173}. These hypotheses remain to be investigated in the future.

Overall, in this chapter it was shown that SS-AFSC sEVs are potent cardioprotective agents when used \textit{in vivo}, without being able to protect directly isolated cardiomyocytes \textit{in vitro}. Moreover, endothelial-mediated cardioprotection was also not observed \textit{in vitro}, thus leaving the mechanism open for further exploration. Nevertheless, the finding that SS-AFSC sEVs are efficiently cardioprotective using the intravenous administration route may prove clinically superior to EVs isolated from other cell types, which are seemingly inefficient when delivered to the bloodstream.
Chapter 6. Proangiogenic effects of SS-AFSC sEVs in vitro

6.1. Introduction

In addition to the cardioprotective functions discussed in the previous chapter, sEVs derived from different cell sources, including MSCs, have been documented to have proangiogenic functions which could provide long-term beneficial effects post MI\(^87\). The proangiogenic effects of MSC sEVs may be mediated via delivery of miRNAs or proteins and multiple candidates have been identified previously (see \(^87\) for review). In spite of the promise that MSC sEVs offer, vesicular purity of the isolates (discussed in detail in section 1.6.2., chapter 3 and chapter 4) is rarely scrutinised which questions the validity of some findings in angiogenesis studies with poor sEV characterisation.

Furthermore, studies designed to compare activity or potency of sEVs derived from different stem cells are also sparse. There are some indications that CPC EVs may be better suited to promote endothelial cell tube formation \textit{in vitro} than bone marrow MSCs but it is not known whether this is translated \textit{in vivo}\(^202\). Additionally, no comparison of foetal MSC sEVs to adult MSCs or cardiac progenitors have been made to date.

There is also little evidence for the effects of foetal MSC sEV on angiogenesis. One study of umbilical cord MSCs used an improved sEV isolation protocol employing a sucrose cushion isolation from serum-free conditioned medium and showed that the sEVs promote dose-dependent proliferation, migration, tube formation and \textit{in vitro} and \textit{in vivo} wound healing by activating Wnt pathway\(^319\). Similarly, placental MSC-derived sEVs were shown to promote migration, proliferation and tube formation of placental microvascular endothelial cells, although this seemed to require exposure of the parent placental MSCs to hypoxia\(^320\). No evidence for a robust proangiogenic effect of AFSC sEVs has been published before, despite the fact that conditioned medium from AFSCs and amniotic MSCs has been demonstrated to have such an effect both \textit{in vitro} and \textit{in vivo}\(^231,233,234\). In fact, limited experimentation indicates that AFSC sEVs may not be potently
proangiogenic in their naïve state\textsuperscript{154,233}. It still remains to be investigated whether the sEVs derived from the SS-AFSC subpopulation of AFSCs can promote angiogenesis and whether these retain the greater benefits reported previously for their parental cell subtype (discussed in section 1.7.).

In this chapter, the \textit{in vitro} proangiogenic effects of SS-AFSC sEV were investigated. Highly-purified and fully-characterised sEV harvests from chapter 4 were used in models of endothelial cell migration, proliferation and tube formation.

6.2. Aims

The aims of this chapter were to:

1) Investigate the potential of SS-AFSC sEVs to promote migration, proliferation and tube formation of endothelial cells \textit{in vitro}

2) Compare the activity of SS-AFSC sEVs to the starting SS-AFSC-conditioned medium for any pronounced effects observed in 1)
6.3. Methods

6.3.1. Endothelial cell migration assays

HUVECs were cultured as explained in section 2.1.2.2. Endothelial cell migration setup is shown in section 2.7 and on Figure 2-5.

An initial experiment was conducted to determine whether the concentration gradient is maintained between top and bottom wells of the chemotaxis chamber. 1.7 mg/ml BSA in PBS (measured by BCA assay) was added to the bottom wells of the chamber and its diffusion to the top wells (containing PBS only) was measured over time by collecting small aliquots every hour and measuring protein concentration using a BCA assay. The concentration in the bottom wells was determined again at 6 hours (i.e. the time selected for subsequent HUVEC migration experiments). The concentration in bottom wells for 1-5 h was estimated using the starting BCA amount and the concentration of BCA measured in the top wells.

A pilot experiment using FBS was carried out to verify that HUVECs can migrate to the bottom chamber in response to a chemoattractant. 10% FBS was used in these experiments. Note that FBS was not always promigratory. This was found to be dependent on the source and lot of the FBS used (data not shown). Comparison between FBS lots and conclusions about its promigratory activity are beyond the scope of this report.

For SS-AFSC sEV dose-response experiments, SS-AFSC sEVs were added to the bottom wells of the chambers at the indicated concentrations. For a comparison between SS-AFSC-conditioned medium and SS-AFSC sEVs, samples with equal protein concentrations (measured by Bradford assays) were added to the bottom wells of the chambers (in varying doses of 20-500 ng/ml). Protein normalisation was carried out in this instance, due to the inclusion of starting conditioned medium which also contains abundant soluble protein.

Two different approaches were taken to determine the importance of sEVs for the promigratory effects of SS-AFSC conditioned medium:

1) a comparison between equal protein concentrations (200 ng/ml) of:
   - starting conditioned medium
- sEV-depleted conditioned medium (including: 10,000 g pellet, \textit{i.e.} large and medium EVs; filtrate from the ultrafiltration step, \textit{i.e.} small proteins, vitamins, aminoacids and ions; and SEC fractions 7.0 ml – 15.0 ml, \textit{i.e.} soluble protein)
- isolated sEVs (pooled SEC fractions 4.0 ml, 4.5 ml and 5.0 ml)

2) a second experiment was conducted whereby ultrafiltration was omitted and conditioned medium was added directly to the column: the ultrafiltration step was found to cause considerable loss of protein (probably due to protein adherence to the membrane filters). Here the following were compared in order to determine whether the promigratory activity is eluted with the sEVs or the soluble protein:
- starting conditioned medium (500 ng/ml)
- sEV fraction of the conditioned medium after SEC (SEC fractions 3.0 ml – 7.0 ml, normalised to the volume of the starting conditioned medium; see Figure 6-3C)
- soluble protein fraction of the conditioned medium after SEC (pooled SEC fractions 7.0 – 15.0 ml, normalised to the volume of the starting conditioned medium; see Figure 6-3C)

6.3.2. Endothelial cell proliferation assays

Details of the assay can be found in section 2.8. SS-AFSC sEVs were added to each well to assess for mitogenic effects. 10% FBS was used as a known mitogen and absorbance results are presented as a percentage of this positive control.

6.3.3. Endothelial cell tube formation assays

Details of the assay can be found in section 2.9. Final concentrations of the treatments were as follows:

1) Control (vehicle for VEGF: 0.0001% (w/v) BSA in PBS)
2) VEGF (25 ng/ml in 0.0001% (w/v) BSA in PBS) (human VEGF_{165}: Cat no 293-VE, R&D systems)
3) Vehicle (for SS-AFSC sEVs, \textit{i.e.} PBS)
4) SS-AFSC sEVs: 3x10^9 particles/ml
5) SS-AFSC sEVs: 1x10^{10} particles/ml
6) SS-AFSC sEVs: 3x10^{10} particles/ml

Six technical replicates (i.e. six wells) were performed for each biological replicate.

6.3.4. Protein content

Protein content was estimated by Bradford assays as explained in section 4.3.5.
6.4. Results

6.4.1. SS-AFSC sEV effects on endothelial cell migration, proliferation and tube formation in vitro

To assess the angiogenic potential of the isolated SS-AFSC sEVs, HUVEC migration, proliferation and tube formation in response to sEVs was studied.

Firstly, to study migration, a modified Boyden’s Chamber assay model was employed. There is a lack of characterisation experiments in the literature to confirm the maintenance of a gradient of chemoattractants across the permeable membrane of the Boyden’s Chamber endothelial chemotaxis assays. To address this, using BSA (molecular weight: 66 kDa), it was established that a stable concentration gradient of nano-sized factors (such as sEVs) can be maintained between the compartments during the chosen time of incubation (Figure 6-1A). This ensures minimal, but large enough diffusion to create a gradient. The effectiveness of a growth-factor rich FBS to drive directional endothelial cell migration was further established, in order to demonstrate the applicability of the model to assay HUVEC migration (Figure 6-1B).

Next, the effects of SS-AFSC sEVs on HUVEC migration were studied. Vesicles promoted migration of endothelial cells in a dose-dependent manner with concentrations of ≥ 3x10⁹ sEVs/ml being effective (p > 0.05 for 1x10⁹ sEVs/ml, p < 0.05 for 3x10⁹ sEVs/ml and p < 0.01 for 1x10¹⁰ sEVs/ml and 3x10¹⁰ sEVs/ml versus Vehicle, Figure 6-2A). sEV promigratory potential was marked and reached levels similar to the FBS used (Figure 6-2A). Intriguingly, this remarkable potency of SS-AFSC sEVs was not observed in models of HUVEC proliferation and tube formation (Figure 6-2B-D). sEVs seemed to weakly promote endothelial cell proliferation, but these effects were subtle compared to the baseline proliferation levels and the FBS control (Figure 6-2B). As expected, in the tube formation assay, VEGF positive control induced capillary formation on the extracellular matrix gel (p < 0.001 VEGF versus Control, Figure 6-2C,D) but this was not observed for any of the doses of SS-AFSC sEVs used (p > 0.05, Figure 6-2C,D).
Figure 6-1. Establishment of a modified Boyden’s Chamber assay of endothelial cell migration

Chemotaxis chamber (NeuroProbe) was used to establish a model of endothelial cell migration. A – Diffusion rate of bovine serum albumin (BSA) was measured for a period of 6 h between the bottom and the top wells of the chemotaxis chamber. BSA (1.7mg/ml, solid black square at 0 h) in PBS was loaded in the bottom wells and PBS in the top wells. Aliquots from the top wells were taken every hour for a total of 6 h and the BSA concentration was measured by BCA assays (black circles). BSA concentration in the bottom chamber was estimated for 1 h – 5 h time points (hollow black squares). The concentration in the bottom chamber was also measured at the end of the incubation period (solid black square at 6 h). Note the maintenance of a concentration difference between the top and the bottom wells after 6 h of incubation. B – HUVEC migration after 6 h of incubation in response to vehicle (PBS) or 10% FBS. Migration is presented as mean staining intensity for each group. ** p < 0.01, Paired Student’s t-test. n = 6.
**Legend on the next page**
Figure 6-2. Effects of SS-AFSC sEVs in models of angiogenesis in vitro (see the previous page)

A – HUVEC migration was assessed using a modified Boyden’s Chamber assay. Cell migration in response to vehicle (PBS), increasing concentrations of SS-AFSC sEVs (1x10⁹-3x10¹⁰ sEVs/ml) or 10% FBS (control) was studied. Migration is presented as mean staining intensity for each group. * p < 0.05, ** p < 0.01, 1-way Repeated Measures ANOVA with Dunnett’s post-hoc test. n = 5. Right panels – representative images of two of the groups (indicated). Scale: 200 µm. B – HUVEC proliferation was assessed using an MTT assay. Cells were plated and incubated for 48 h in presence of vehicle (PBS) or increasing concentrations of SS-AFSC sEVs (3x10⁸-1x10¹⁰ sEVs/ml). Proliferation is presented relative to a positive control of 10% FBS. * p < 0.05, ** p < 0.01, 1-way Repeated Measures ANOVA with Dunnett’s post-hoc test. n = 7. C,D – In vitro angiogenesis was assessed using a HUVEC tube formation assay. Cells were plated on Geltrex™ (extracellular matrix gel) and incubated for 16 h in absence of treatments (Control) or in presence of VEGF (25 ng/ml), vehicle (PBS) or increasing concentrations of SS-AFSC sEVs (3x10⁸-3x10¹⁰ sEVs/ml). Results are presented in panel C as number of tubes per mm². *** p < 0.001, ns – non-significant (p > 0.05), 1-way Repeated Measures ANOVA with Tukey’s post-hoc test. n = 3. Representative images are shown in panel D. Scale: 200 µm.

6.4.2. Comparison of SS-AFSC sEV and starting conditioned medium effects on endothelial cell migration in vitro

Similar to the isolated SS-AFSC sEVs, SS-AFSC-conditioned medium demonstrated dose-dependent promigratory effects on endothelial cells (Figure 6-3A). sEVs promoted migration at doses starting from 50 ng/ml while at least 100 ng/ml of conditioned medium were necessary to stimulate HUVEC migration (Figure 6-3A). At higher doses of ≥ 250 ng/ml, however, conditioned medium was more effective than sEVs (Figure 6-3A). Pharmacologically speaking, this indicates that sEVs may have higher potency while the conditioned medium possesses better efficacy. However, current experiments cannot confirm the potency in terms of EC₅₀ values since a full dose-response curve was not generated and the aforementioned argument should be verified in subsequent studies.

Next, the effects of the SS-AFSC sEV were more directly compared to those observed with the starting SS-AFSC-conditioned medium. Intriguingly, the promigratory effects of the conditioned medium were completely lost when
it was depleted of sEVs, indicating that vesicles are the active chemotactic mediator of the SS-AFSC secretome (p < 0.001 for Conditioned medium versus sEV-depleted conditioned medium and p > 0.05 for Conditioned medium versus isolated sEVs, Figure 6-3B). It should be noted however, that sEV-depleted conditioned medium consisted of pooled pellets and filtrates obtained from the sEV purification process (see Figure 2-2) and as indicated in chapter 4, there is a significant loss of particles and protein during the procedure, which may confound the comparison of sEVs with conditioned medium. Therefore, to directly compare the sEV-rich and sEV-poor (i.e. soluble protein-rich) fraction of the SS-AFSC-conditioned medium, an aliquot of it was run on a SEC column (omitting the ultrafiltration step to eliminate confounders), and sEV-rich fractions (3.5 ml – 7.0 ml) were separated from soluble protein fractions (7.0 ml – 15.0 ml) (Figure 6-3C). Conditioned medium was then directly compared to its sEV-rich and protein-rich fractions which demonstrated that sEV application is sufficient to recapitulate the effects of the SS-AFSC-conditioned medium (p > 0.05 for Conditioned medium versus sEV-rich fraction and p < 0.05 for Conditioned medium versus Protein fraction, Figure 6-3C).

Overall, SS-AFSC sEVs had minor effects on proliferation and tube formation of endothelial cells but they potently stimulated endothelial cell migration in vitro. Importantly, despite the complexity of the soluble protein secretome of SS-AFSCs, sEVs were found to be the active promigratory factor of the SS-AFSC-conditioned medium.
Figure 6-3. Comparison of the promigratory effects of starting SS-AFSC-conditioned medium and isolated sEVs

A – HUVEC migration in response to different doses of SS-AFSC-conditioned medium (CM) or isolated SS-AFSC sEVs (20-500 ng/ml). Migration is presented as raw intensity values. ** p < 0.01 vs Vehicle, *** p < 0.001 vs Vehicle, $ p < 0.001 vs CM (respective dose), 1-way Repeated Measures ANOVA with Tukey’s post-hoc test. n = 5. B – HUVEC migration in response to SS-AFSC-conditioned medium (CM), SS-AFSC conditioned medium depleted of sEVs (CM-sEV-free) or isolated SS-AFSC sEVs (all at 200 ng/ml). Migration is presented as raw intensity values. *** p < 0.001, ns – non-significant (p > 0.05), 1-way Repeated Measures ANOVA with Dunnett’s post-hoc test. n = 4. C – HUVEC migration in response to SS-AFSC-
conditioned medium (CM) or different SEC fractions of CM: sEVs (3-7 ml) or Protein (7-15 ml). CM was used at 500 ng/ml while sEVs and protein were normalised to the CM volume. Representative SEC separation is presented on the small panel on the right taken from Figure 4-4. Note that promigratory activity was eluted in the sEV fraction. Migration is presented as raw intensity values. * p < 0.05, *** p < 0.001, 1-way Repeated Measures ANOVA with Tukey’s post-hoc test. n = 6.
6.1. Discussion

In this chapter it was shown that SS-AFSC sEVs act as potent chemoattractant factors for endothelial cells, stimulating their migration in a dose-dependent manner. No pronounced effects on endothelial cell proliferation or tube formation in vitro were recorded, implying that SS-AFSC sEVs have specific actions on endothelial cells and may only partially impact angiogenesis. Intriguingly, SS-AFSC-conditioned medium also promoted endothelial cell migration dose-dependently, while its depletion of sEVs abolished these effects. Furthermore, experiments with conditioned medium separated by SEC on sEV-rich and sEV-poor/protein-rich fractions showed definitively that promigratory activity of the SS-AFSC-conditioned medium elutes primarily in the sEV fraction.

There is some emerging evidence for AFSC sEV role in angiogenesis that was published during the course of this project, but it has been largely inconsistent and ill-defined mechanistically. Poor characterisation of the obtained sEV samples dominated some of these reports as well as the use of non-standardised protocols for sEV isolation. Moreover, the subtype of AFSCs used as an sEV source was not indicated in these investigations.

Nevertheless, Balbi et al. argued in two different studies that AFSC sEVs are not potent stimulators of angiogenesis in vivo. In one of their reports, there was some indication that after hypoxic incubation of the AFSCs the released vesicles may be able to increase vascular formation in sEV-loaded Matrigel plugs implanted subcutaneously in mice as shown by the haemoglobin content in the plugs. However, this was not seen when Matrigel plugs were loaded with sEVs derived from naïve AFSCs. In their more recent study, the same group similarly showed that angiogenesis following permanent MI in mice can be induced by AFSC-conditioned medium but following ultracentrifugation separation, both sEV fraction and non-sEV fraction of the medium lost their proangiogenic properties. Interestingly, the in vitro findings presented in the current report somewhat support the previous observations of Balbi et al. in vivo. It was demonstrated here that SS-AFSC sEVs promote migration of endothelial cells but do not considerably affect
proliferation and tube formation. The minor but statistically significant effect on HUVEC proliferation is unlikely to be biologically important since the effect was markedly lower than that for the serum positive control. Indeed, medium conditioned by SS-AFSCs was previously shown to promote migration, proliferation and tube formation of umbilical cord endothelial colony forming cells but the most pronounced effects were recorded for its promigratory activity.

Using two different approaches, it was demonstrated here that sEVs carry the chemotactic function of the SS-AFSC-conditioned medium. Given that Balbi et al. observed effects of conditioned medium but not of the isolated sEVs or the sEV-depleted conditioned medium and argue that the presence of both soluble secretome and released sEVs is required to achieve efficient angiogenesis, it is possible that the sEVs are the active promigratory component of the SS-AFSC-conditioned medium while the non-EV SS-AFSC secretome may be required for other angiogenic processes (e.g. proliferation, tube formation). This is currently unknown, and it will be interesting to investigate in the future the activity of the SS-AFSC-conditioned medium in the models of endothelial cell proliferation and tube formation and to compare it to its promigratory effects.

It has previously been demonstrated that various progenitor cell EVs stimulate angiogenic processes in vitro including EVs derived from adult BM-MSCs, CPCs, CDCs, iPSCs and even foetal MSCs (i.e. umbilical cord MSCs). It is possible that the lack of potent proangiogenic effects on proliferation and tube formation shown here is characteristic to the SS-AFSC-derived vesicles. Alternatively, this may be due to a specific feature of the sEV population obtained by the isolation protocol used here or due to the superior purity of the SS-AFSC sEVs in comparison to previous reports.

Target endothelial cell type may also be responsible for the discrepant results. HUVEC migration assays are widely used in vitro and as venous cells they are well-suited for studies of angiogenesis which is more prominent when originating from a vein as opposed to an artery explant. In fact, most of the angiogenesis studies with EVs cited above use HUVECs but there are also investigations looking into vesicular proangiogenic effects in populations of cardiac endothelial cells. The latter may be more appropriate
to use in future experiments since endothelial cells specific to the heart, such as primary cardiac left ventricular microvascular endothelial cells\textsuperscript{308}, will have greater relevance to proangiogenic processes in the setting of MI. Furthermore, systematic comparative studies between sEVs originating from different progenitor cell types and also between different sEV isolation procedures are required to draw definitive conclusions about the effects of SS-AFSC sEVs on angiogenesis.

The finding that SS-AFSC sEVs are very efficient in promoting migration of endothelial cells but only modestly affect proliferation and do not stimulate tube formation is intriguing and quite unusual. A limitation of this study is the lack of data on \textit{in vivo} angiogenesis. Migration, proliferation and tube formation are the basis of \textit{in vivo} angiogenesis but processes such as matrix degradation, extracellular matrix deposition, pericyte recruitment and vascular remodelling are also crucial in a whole organism\textsuperscript{79,324}. Therefore, one cannot definitively conclude about the \textit{in vivo} effects of SS-AFSC sEVs on angiogenesis, especially in the setting of MI. Hence, it can be speculated that SS-AFSC sEVs may only act on part of the angiogenic process. For instance, they may stimulate tip cells migration without affecting stalk cell proliferation of a newly forming capillary\textsuperscript{325}. Certain chemotactic factors with no mitogenic activity have been reported previously such as angiopoietin-1 which promotes migration of HUVECs but does not stimulate proliferation\textsuperscript{326,327}, despite activating its receptor TIE2\textsuperscript{326}. Angiopoietins were not detected by proteomic analysis in the SS-AFSC sEV samples in this report (see chapter 7) but it has to be noted that LC-MS/MS approaches suffer from the inability to detect all proteins in a sample due to the high abundance of a handful of species. Therefore, the presence of angiopoietins could not be completely excluded, and the protein arrays performed here (see chapter 7) demonstrated that low levels of angiopoietins may be present in the sEVs. This remains to be investigated further using targeted approaches (\textit{e.g.} immunoblotting). It is also plausible that another chemotactic factor without mitogenic activity is responsible for the promigratory effects observed here.

Given the role of SS-AFSC sEVs as chemoattractants and the increasing amount of literature published on the effects of sEVs on immune cells in the setting of MI\textsuperscript{67,280,328} as well as their general immunomodulatory effects\textsuperscript{329}, it is
also plausible that this chemotactic response may be important for immune cell mobilisation and recruitment. Future studies are expected to investigate this hypothesis in detail.

Overall, in this chapter it was shown that SEC-isolated SS-AFSC sEVs promote migration of endothelial cells dose-dependently with only minor effects on endothelial cell proliferation and tube formation. Interestingly, the promigratory effects of SS-AFSC-conditioned medium were recapitulated by the use of isolated SS-AFSC sEVs, indicating that vesicles are the most potent chemotactic component in the SS-AFSC secretome.
Chapter 7. SS-AFSC sEV cargo and the mechanisms of the promigratory effects of the sEVs

7.1. Introduction

Deciphering of the mechanisms of the promigratory effects of SS-AFSC sEVs observed in chapter 6 is crucial to understand their overall role in angiogenesis, but also other processes related to cell migration such as immune cell recruitment. The two main mechanistic questions that need to be answered are related to the cargo in the SS-AFSC sEVs responsible for the observed effects and the signalling in the target endothelial cells that mediates them.

The cardioprotective and promigratory activities of SS-AFSC sEVs were observed in an acute setting here (2 h and 6 h, respectively). Hence, signalling cascade activation by protein cargo in the sEV was deemed to be the most likely mechanistic explanation, rather than translational regulation by sEV-contained nucleic acids, e.g. miRNAs. Hence, in this chapter, attention was focussed on the protein cargo of the vesicles. It is noteworthy that vesicular miRNA can also have long-term activity as discussed later in this report.

Multiple design strategies could be employed to investigate the involvement of the sEV cargo in endothelial cell migration. For instance, a more biased way could be based on a literature-informed experimental approach, focussing on a protein known to be abundant in the sEVs and to mediate a specific function. Such an example is the study conducted by our group (discussed in section 1.6.3.) which identified HSP70 as a potential mediator of plasma sEV-delivered cardioprotection: a hypothesis stemming from the well-known presence of HSP70 protein in the vesicles and the powerful cardioprotective potential of heat shock proteins\(^{64}\). Despite this success, sEVs possess incredibly rich repertoire of proteins. Hence, a more detailed study of the abundancy of the proteins in the sEV samples may provide a better starting point for mechanistic experimentation. Common approaches for this include protein profiling by membrane arrays or LC-MS/MS proteomics analysis.
Protein array profiling is a relatively simple and less expensive technique than proteomics. It has previously been employed in investigations of the secretome of various MSC types330–334, including foetal MSCs330,332,335,336, and even SS-AFSCs231. It should be noted, however, that protein arrays can only study a handful of proteins and can also be considered biased towards a selected panel of factors. Nevertheless, this approach has previously provided useful information about the functional potential of the MSC secretome231,330–336 but also about MSC sEV proteome335. For instance, one report showed a correlation exists between expression of proangiogenic factors in MSC sEVs harvested from different donors and their potency to drive endothelial cell migration and tube formation in vitro and angiogenesis in Matrigel plugs in vivo335. This indicates that protein array profiling may provide a useful basis for investigation of proangiogenic mechanisms.

An alternative method to protein arrays is proteomics which provides a more complete picture of the whole proteome of a sample. The secretome of adult MSCs and foetal MSCs337,338, including AFSCs (non-selected for SS-AFSCs)236, has already been investigated previously. There is even one study that reported a mass spectrometry analysis of AFSC sEVs236. However, as discussed in section 4.1., the conditions of cell incubation for sEV isolation in this report involved prolonged maintenance of the cells in saline solution which may have induced cell rupture and non-specific release of intracellular milieu236. This may explain the detection of a large proportion of peptides related to non-sEV structures such as proteins involved in translation236.

With regard to angiogenic processes, one comparative proteomic and functional study showed that bone marrow and umbilical cord-derived MSCs contain a more complete angiogenesis-related proteome than adipose tissue MSCs that also translated to a more pronounced promigratory activity on monocytes, a more efficient differentiation into macrophages (associated with a proangiogenic response) and a bigger increase of angiogenesis-related membrane markers in HUVECs337. Therefore, similar to the protein array profiling, proteomics may also prove useful for identifying proangiogenic mediators. The power of this approach is evidenced by a recent study in CPC sEVs202. Barile et al. found PAPPA (pregnancy-associated plasma protein A) to be enriched in CPC sEVs compared to bone marrow MSC sEVs by
proteomic analysis and showed that PAPPA is expressed on the sEV membrane by immunogold labelling\textsuperscript{202}. By a series of mechanistic experiments Barile \textit{et al.} identified PAPPA as a crucial mediator for the more potent effects of CPC sEV compared to bone marrow MSCs such as cytoprotection and improvement of heart function in a model of permanent MI in rats \textit{in vivo}\textsuperscript{202}.

sEVs can deliver their protein or nucleic acid cargo to the cytoplasm of the recipient endothelial cells through endocytosis or cell membrane fusion\textsuperscript{91,117} (studied and discussed in chapter 8). Alternatively, membrane sEV proteins may activate receptors on the endothelial cell surface to trigger intracellular signalling cascades\textsuperscript{87,91,117}. In this chapter the latter was studied with a particular focus on kinase pathways since a plethora of kinases are involved in endothelial cell migration, \textit{e.g.} PI3K, Src, Fyn, Yes, FAK, MYLK, p38\textsuperscript{339–342}. Interestingly, there are some indications that MSC sEVs activate kinase or kinase-regulated pathways such as PI3K\textsuperscript{191}, NF-\kappa B\textsuperscript{343}, ERK\textsuperscript{344} and STAT3\textsuperscript{344} to mediate proangiogenic effects. However, the evidence for this is sparse, some studies were performed with cells of a non-endothelial nature, and sometimes they only indirectly inferred activation of the aforementioned pathways. Furthermore, it remains unknown which signalling cascades are specifically responsible for endothelial cell migration.

In this chapter, the mechanisms of SS-AFSC sEV promigratory effects were studied. Guided by protein arrays and proteomic analysis, inhibitors of some of the most abundant factors in the SS-AFSC sEV cargo were used in endothelial cell migration assays to identify potential key players. Furthermore, employing a more specific western blotting or a broader approach by protein kinase arrays, signalling pathways in the endothelial cells in response to SS-AFSC sEVs were investigated.

\textbf{7.2. Aims}

The aims of this chapter were to:

1) Attempt to determine the potential mediator of the SS-AFSC sEV-promoted endothelial cell migration using protein arrays and proteomics data of the sEV harvests as guidance
2) Investigate the involvement of endothelial PI3K and other kinase signalling pathways in the SS-AFSC sEV-promoted endothelial cell migration
7.3. Methods

7.3.1. Protein arrays

Commercially available protein array kits were obtained from R&D Biosciences (see section 2.12.). Manufacturer’s instructions were followed with some modifications.

For SS-AFSC-conditioned medium and SS-AFSC sEV protein profiling, each membrane was blocked using array buffers (provided by the manufacturer). ~15 µg protein for Cytokine Arrays and ~10 µg protein for Angiogenesis Arrays were lysed with 0.1% (v/v) Triton X-100 and vortexing for 30 s\(^{251}\) (for both SS-AFSC-conditioned medium and SS-AFSC sEVs). Samples were mixed with biotinylated antibody detection cocktail (provided by the manufacturer) and incubated on the membranes overnight at 4°C. To optimise sensitivity, the streptavidin-HRP conjugates of the kit were replaced by 250 ng/ml streptavidin-DyLight 800 conjugate for detection of biotin-conjugated antibodies (21851, ThermoFisher). Membranes were imaged, and densitometry was performed on Odyssey system (LI-COR). The membrane spot coordinates can be found on https://resources.rndsystems.com/pdfs/datasheets/ary007.pdf and https://resources.rndsystems.com/pdfs/datasheets/ary005b.pdf. Spot intensities were normalised to reference control spots and presented as relative pixel intensities.

To profile the phosphorylation of intracellular signalling kinase pathways, HUVECs were pre-starved for 3 h in DMEM (ThermoFisher) supplemented with 25 mM glucose, 4 mM GlutaMAX, 50 units/ml penicillin (Sigma), 50 µg/ml streptomycin (Sigma). Cells were then treated with vehicle (PBS) or sEVs (1x10\(^{10}\) particles/ml) for 15 min or 3 h in the starvation medium. This was followed by immediate \textit{in situ} lysis using the buffer provided in the kit (already containing phosphatase inhibitors) further supplemented with protease inhibitors (1861279, ThermoFisher). Lysates were rocked for 30 min at 4°C for complete breakdown of the cells and centrifuged for 5 min at 14,000 g. Pellets were discarded and the supernatant collected and stored at -80°C. ~300 µg protein mixed with biotinylated antibody detection cocktail was loaded on each
membrane set after blocking the membrane with array buffers (all provided by the manufacturer). To optimise sensitivity, the streptavidin-HRP conjugates of the kit were replaced by 250 ng/ml streptavidin-DyLight 800 for detection of biotin-conjugated antibodies (21851, ThermoFisher). Membranes were imaged, and densitometry was performed on Odyssey system (LI-COR). The membrane spot coordinates can be found on
https://resources.rndsystems.com/pdfs/datasheets/ary003b.pdf.
Spot intensities were normalised to control protein spots (β-catenin or HSP60) and presented as relative pixel intensities.

7.3.2. Proteomics

Functional enrichment analysis was performed as per section 4.3.8. Proteins detected in the sEV samples (Figure 7-2) or proteins enriched in or exclusive to the sEV samples (Figures 7-3, 7-4, 7-5) were used, excluding those assigned a minimum LFQ value (see section 2.3.6.) and those with < 2 PSMs.

Additionally, FunRich software version 3.1.3.345 was used to build a Venn diagram and STRING database online tool version 11.0346 was used to produce a network of SS-AFSC sEV-enriched and SS-AFSC sEV-exclusive proteins (the highest confidence setting was chosen with interaction score of ≥ 0.900; disconnected nodes were hidden). sEV enrichment was defined as > 1.5 times (log2 > 0.58) higher LFQ peptide area in the sEV sample compared to the conditioned medium sample. Complete analysis can be found on https://drive.google.com/open?id=1fUyiEZNi_FKIXOCqjBNOBF8VN_kLDa6f.

7.3.3. Endothelial cell migration

HUVECs were cultured as explained in section 2.1.2.2. Details of the endothelial cell migration setup can be found in section 2.7.

SS-AFSC sEVs or human recombinant SDF1α (130-093-997, Miltenyi Biotec) were added to the bottom wells of the chemotaxis chamber at the indicated concentrations. Pharmacological inhibitors (GDC-0941: SM19-10, Cell Guidance Systems at 100 nM; AMD3100: 3299, Tocris Bioscience at 10
µM; TAK-242: 614316, Calbiochem at 10 µM) or antibodies (anti-PTX3, Clone EPR18678-105, Abcam at 1 µg/ml; anti-RTN4B, Clone AF6034, R&D Systems at 1 µg/ml or 5 µg/ml) were added to both top and bottom wells of the chamber where indicated.

7.3.4. Western blotting

Primary antibodies used for western blotting are shown in Table 3. Secondary antibodies were goat anti-mouse IgG (926-68070, LI-COR) and goat anti-rabbit IgG (926-32211, LI-COR).

Table 7-1. Antibodies used for Western blotting analysis of signalling pathways in HUVECs

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Species</th>
<th>Cat no</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>total-AKT</td>
<td>Mouse</td>
<td>2920</td>
<td>Cell Signalling Technology</td>
</tr>
<tr>
<td>phospho-S473-AKT</td>
<td>Rabbit</td>
<td>4060</td>
<td>Cell Signalling Technology</td>
</tr>
<tr>
<td>total-PRAS40</td>
<td>Rabbit</td>
<td>2610</td>
<td>Cell Signalling Technology</td>
</tr>
<tr>
<td>phospho-T246-PRAS40</td>
<td>Rabbit</td>
<td>2997</td>
<td>Cell Signalling Technology</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Mouse</td>
<td>sc-32233</td>
<td>Santa Cruz Biotechnology</td>
</tr>
</tbody>
</table>
7.4. Results

7.4.1. SS-AFSC sEV cargo

The preceding chapter demonstrated that SS-AFSC sEVs promote migration of endothelial cells. Therefore, to obtain a profile of potential promigratory factors present in the SS-AFSC sEVs that may be responsible for the observed effects, two different strategies were used here: protein arrays and proteomic analysis.

Multiple potential proangiogenic factors were found to be present in the conditioned medium and the sEV isolates by angiogenesis and cytokine protein arrays (Figure 7-1A,B). Some factors such as PTX3, were clearly enriched in the isolated SS-AFSC sEV samples, while others such as PAI1, TIMP1 and TSP1, were mostly found in the SS-AFSC-conditioned medium (Figure 7-1A,B). The most abundant angiogenic factors in the sEVs were PTX3, EDN1 and DPPIV while many others were also found at lower levels (e.g. VEGF, ANGPT2) (Figure 7-1A). When examining protein expression using a cytokine array, many of the cytokines were identified, although none were present at particularly high levels in the sEVs. The most abundant ones in the sEVs were MIF and SDF1 (Figure 7-1B).

Proteomic analysis mostly confirmed the observations from the protein arrays with some exceptions (identified proteins can be accessed on https://drive.google.com/open?id=1fUyiEZNi_FKiXOCqiBNOBF8VN_kLDa6f) . This approach identified further potential promigratory mediators in the SS-AFSC sEV samples such as BGN\textsuperscript{347} and RTN4\textsuperscript{264}, which were enriched in the sEV samples compared to the starting SS-AFSC-conditioned medium. 484 proteins were identified in the sEV samples and 232 proteins were identified in the conditioned medium samples using the exclusion criteria detailed in sections 2.3.6., 4.3.8. and 7.3.2. (Figure 7-2). 331 proteins were detected at higher relative levels in the SS-AFSC sEV samples and most of those were exclusive to the sEV isolates (Figure 7-2 and https://drive.google.com/open?id=1fUyiEZNi_FKiXOCqiBNOBF8VN_kLDa6f) .
Since sEVs appeared to mediate the chemotactic effects of the SS-AFSC-conditioned medium (see section 6.4.2.), the attention was then focussed on the sEV-exclusive and sEV-enriched (> 1.5 times) proteins in the SS-AFSC sEV harvests in comparison to the SS-AFSC-conditioned medium. Biological Process classification yielded numerous overrepresentations in the sEV proteome including terms related to vesicle-mediated transport, membrane localisation, and even viral and immune processes (likely due to the high abundance of vesicle-associated proteins) (Figure 7-3A and https://drive.google.com/open?id=1fUyiEZNi_FKIXOCqiBNOBF8VN_kLDa6f). Proteins associated with cell migration were also highly overrepresented in the sEV samples: “positive regulation of locomotion”: $p = 2.7 \times 10^{-10}$, 3.9-fold enrichment; “positive regulation of cell adhesion”: $p = 7.5 \times 10^{-9}$, 4.3-fold enrichment; “positive regulation of cell motility”: $p = 2.0 \times 10^{-8}$, 3.7-fold enrichment; “positive regulation of cell migration”: $p = 1.2 \times 10^{-7}$, 3.6-fold enrichment; “positive regulation of cytoskeleton organisation”: $p = 3.4 \times 10^{-6}$, 5.0-fold enrichment; “positive regulation of actin filament bundle assembly”: $p = 4.1 \times 10^{-2}$, 6.9-fold enrichment (Figure 7-3B). These were distinct from any probable contaminants such as ribosomal proteins and histones which clustered elsewhere (see Figure 7-4 and https://drive.google.com/open?id=1fUyiEZNi_FKIXOCqiBNOBF8VN_kLDa6f). KEGG pathway analysis also showed enrichment in pathways which may be associated with vesicular trafficking and cell migration and it also indicated the presence of ribosome-associated pathways (likely due to the observed low level of cell death in the SS-AFSC cultures and the enrichment of ribosomal components in the process of sEV isolation – see sections 4.5. and 7.5. for discussion) (Figure 7-5). Interestingly, there was a significant enrichment of proteins associated with the PI3K-AKT signalling pathway, known to be involved in endothelial cell migration\textsuperscript{339,340} (Figure 7-5; $p = 4.1 \times 10^{-4}$, 2.7-fold enrichment).

Overall, SS-AFSC sEVs carried diverse proteins, some of which may be associated with the promigratory effects observed in the previous chapter. Intriguingly, some of the abundant sEV-associated proteins, such as including SDF\textsuperscript{348}, MIF\textsuperscript{349}, PTX\textsuperscript{350}, RTN4\textsuperscript{264} and BGN\textsuperscript{347}, have previously been shown to exhibit chemotactic activities.
Figure 7-1. Cytokine and angiogenic factor cargo of SS-AFSC-conditioned medium and SS-AFSC sEVs

A,B – Protein arrays for detection of angiogenic factors (A) and cytokines (B) performed on SS-AFSC-conditioned medium or SS-AFSC sEVs after vesicle lysis. Quantification of relative levels (relative to control spots at the top left, bottom left and top right corners of each membrane) of 55 angiogenic factors (A) and 36 cytokines (B) shown together with images of the array membranes. See section 2.12. and 7.3.1. for details about the procedure.
Figure 7-2. Proteins in SS-AFSC-conditioned medium and SS-AFSC sEVs

Venn diagram depicting the number of proteins in the SS-AFSC-conditioned medium and SS-AFSC sEVs. 232 proteins were detected in the SS-AFSC-conditioned medium (blue) and 484 proteins were identified in the SS-AFSC sEVs (red) by LC-MS/MS. 71 proteins were exclusive to the SS-AFSC-conditioned medium sample while 323 proteins were exclusive to the isolated SS-AFSC sEVs.

See https://drive.google.com/open?id=1fUyiEZNi_FKlXOCqjBNOBF8VN_kLDa6f for a full list of proteins.
A

- localization
- vesicle-mediated transport
- transport
- establishment of localization
- protein localization to membrane
- protein localization
- cellular protein localization
- cellular macromolecule localization
- cellular localization
- macromolecule localization
- cellular component biogenesis
- cellular component assembly
- establishment of protein localization
- cellular component organization or biogenesis
- export from cell
- regulation of localization
- symbiont process
- cellular component organization
- viral process
- interspecies interaction between organisms
- secretion by cell
- nitrogen compound transport
- intracellular transport
- protein transport
- secretion
- peptide transport
- exocytosis
- neutrophil mediated immunity
- amide transport
- neutrophil degranulation
- establishment of localization in cell
- neutrophil activation involved in immune response
- establishment of protein localization to membrane
- neutrophil activation
- movement of cell or subcellular component
- granulocyte activation
- locomotion
- positive regulation of cell component organization
- myeloid leukocyte mediated immunity
- organic substance transport
- leukocyte degranulation
- protein localization to endoplasmic reticulum
- vesicle organization
- establishment of protein localization to endoplasmic reticulum
- myeloid cell activation involved in immune response
- cell activation
- multi-organism process
- cell activation involved in immune response
- establishment of protein localization to organelle
- cell adhesion

Number of proteins

*Legend on the next page*
Figure 7-3. Gene ontology (GO) Biological Process terms overrepresented in SS-AFSC sEVs

Proteomic analysis of SS-AFSC sEV-enriched proteins (> 1.5 times) and SS-AFSC sEV-exclusive proteins was performed. A – Top 50 Gene ontology (GO) Biological Process terms (by p values for enrichment) overrepresented in the SS-AFSC sEVs. See the previous page. B – GO Biological Process terms overrepresented in the SS-AFSC sEVs and associated with cell migration. In both panels terms are ranked by their p values as calculated by g:Profiler (see sections 4.3.8. and 7.3.2. for details). Red bars show the expected number of proteins in each GO term group (based on the number of proteins in the human proteome and the query size) and blue bars show the observed number of proteins associated with each GO term. See full results on https://drive.google.com/open?id=1fUyiEZNi_FKIXOCqjBN0BF8VN_kLDa6f.
Figure 7-4. STRING interaction network of proteins enriched in or exclusive to the SS-AFSC sEVs

Protein interaction network of SS-AFSC sEV-enriched proteins (> 1.5 times) and SS-AFSC sEV-exclusive proteins. Protein IDs are shown, and disconnected nodes are hidden. Proteins in red shading are associated with the Gene Ontology Biological Process term “positive regulation of locomotion” (p = 2.7x10^{-10}, 3.9-fold enrichment).
Figure 7-5. KEGG pathways overrepresented in SS-AFSC sEVs
Proteomic analysis of SS-AFSC sEV-enriched proteins (> 1.5 times) and SS-AFSC sEV-exclusive proteins was performed. All KEGG pathways with significant functional enrichment are presented in the graph and they are ranked by their p values calculated by g:Profiler (see sections 4.3.8. and 7.3.2. for details). Red bars show the expected number of proteins in each KEGG pathway (based on the number of proteins in the human proteome and the query size) and blue bars show the observed number of proteins associated with each KEGG pathway. See full results on https://drive.google.com/open?id=1fUyiEZNi_FKIXOCqjBNOBF8VN_kLĐa6f.
7.4.2. SS-AFSC sEV promigratory effects: mediators in the SS-AFSC sEV cargo

To investigate the mechanism of sEV-induced endothelial cell migration, pathway inhibitors of some abundant factors identified in the SS-AFSC sEVs by protein arrays or proteomic analysis were used. These included an anti-PTX3 antibody, an inhibitor of the SDF1- and MIF-activated CXCR4 receptor, an inhibitor of BGN-activated TLR4 receptor, and an anti-RTN4B antibody. Application of an anti-PTX3 antibody had no effect on the SS-AFSC sEV-induced migration (p > 0.05, sEVs versus sEVs + anti-PTX3; Figure 7-6A). A CXCR4 inhibitor efficiently blocked exogenous SDF1α-mediated migration (p < 0.01, SDF1α versus SDF1α + AMD3100; Figure 7-6B) but it did not reduce significantly sEV-induced endothelial chemotaxis (p > 0.05, sEVs versus sEVs + AMD3100; Figure 7-6C). Similarly, an inhibitor of TLR4 had no effect on endothelial cell migration stimulated by SS-AFSC sEVs (p > 0.05, sEVs versus sEVs + TAK-242; Figure 7-6D). Finally, application of an antibody against RTN4B also had no effect on the sEV-induced endothelial cell chemotaxis (p > 0.05, sEVs versus sEVs + anti-RTN4B (for both 1 µg/ml and 5 µg/ml); Figure 7-6E).

Overall, several sEV cargo proteins were excluded as potential mediators of the SS-AFSC sEV-promoted endothelial cell migration. Further studies are required to precisely identify the players in this process.
Figure 7-6. Mechanism of SS-AFSC sEV-induced endothelial cell migration: mediators in the sEV cargo

HUVEC migration in response to SS-AFSC sEVs in combination with various inhibitors or antibodies. A – PTX3 does not mediate SS-AFSC sEV-induced HUVEC migration. Anti-PTX3 – antibody against PTX3. * p < 0.05, ns – non-significant (p > 0.05), 1-way Repeated Measures ANOVA with Tukey’s post-hoc test. n = 4. B – Exogenous SDF1α stimulates HUVEC migration via CXCR4 activation. AMD3100 – CXCR4 inhibitor. * p < 0.05, ns – non-significant (p > 0.05), 1-way Repeated Measures ANOVA with Tukey’s post-hoc test. n = 8. C – CXCR4 does not mediate SS-AFSC sEV-induced HUVEC migration. *** p < 0.001, ns – non-significant (p > 0.05), 1-way Repeated Measures ANOVA with Tukey’s post-hoc test. n = 5. D – TLR4 does not mediate SS-AFSC sEV-induced HUVEC migration. TAK-242 – TLR4 inhibitor. ** p < 0.01, ns – non-significant (p > 0.05), 1-way Repeated Measures ANOVA with Tukey’s post-hoc test. n = 4. E – RTN4B does not mediate SS-AFSC sEV-induced HUVEC migration. Anti-RTN4B – antibody against RTN4B. * p < 0.05, ns – non-significant (p > 0.05), 1-way Repeated Measures ANOVA with Tukey’s post-hoc test. n = 4.
7.4.3. SS-AFSC sEV promigratory effects: signalling effectors in the target endothelial cells

Mechanistically, the PI3K pathway is a known player in developmental vascular formation\textsuperscript{339}, post MI angiogenesis\textsuperscript{340} and endothelial cell migration\textsuperscript{339,340}, and proteins associated with the PI3K-AKT pathway were shown here to be significantly overrepresented in the sEV proteome (see section 7.4.1. and Figure 7-5). Intriguingly, inhibiting the PI3K pathway in endothelial cells reduced SS-AFSC sEV-stimulated migration by 54 ± 15\% (p < 0.001, sEVs versus sEVs + GDC-0941; Figure 7-7A). However, administration of sEVs (3x10\textsuperscript{9} sEVs/ml) led to only a subtle, non-significant increase in phosphorylation of AKT, a downstream PI3K target (p > 0.05, Vehicle versus sEVs; Figure 7-7B). The same non-significant increase was seen when using higher sEV concentration (1x10\textsuperscript{10} sEVs/ml; Figure 7-7C) or additional downstream PI3K targets (i.e. PRAS40; Figure 7-7D).

To provide a broader analysis of potential downstream phosphorylation of kinases or their targets in the recipient endothelial cells, a phospho-kinase array profiling was performed after treatment of HUVECs with SS-AFSC sEVs. Array membrane analysis showed no increase in phosphorylation of 43 different signalling kinases or kinase targets in the endothelial cells after either a 15-min short-term (Figure 7-8A) or a 3-h long-term (Figure 7-8B) incubation of HUVECs with SS-AFSC sEVs.

Overall, SS-AFSC sEV-promoted endothelial cell migration required, but was not solely dependent on, PI3K signalling in the target endothelial cells.
Figure 7-7. Mechanism of SS-AFSC sEV-induced endothelial cell migration: involvement of endothelial PI3K pathway

A – HUVEC migration in response to SS-AFSC sEVs and vehicle (PBS, DMSO) or PI3K pathway inhibitor (GDC-0941) at the indicated concentrations. *** p < 0.001, ** p < 0.01, ns – non-significant (p > 0.05), 1-way Repeated Measures ANOVA with Tukey’s post-hoc test. n = 5. B – Western blotting results for total and phosphorylated AKT in endothelial cells. HUVECs were incubated with Vehicle (PBS, DMSO), GDC-0941 (100 nM) SS-AFSC sEVs (3x10^9 particles/ml), SS-AFSC sEVs + GDC-0941 or insulin (100 nM, positive control) for 15 min. Results are presented relative to 500 nM insulin control. *** p < 0.001, ns – non-significant, 1-way Repeated Measures ANOVA with Tukey’s post-hoc test. n = 6. Representative images shown on the right. p-AKT: phosphorylated AKT, t-AKT: total AKT.

C,D – Western blotting results for total and phosphorylated AKT and PRAS40 in HUVECs – same as per panel B but with a higher concentration of SS-AFSC sEVs (1x10^10 particles/ml). Panel C: *** p < 0.001, ns – non-significant, 1-way Repeated Measures ANOVA with Tukey’s post-hoc test. n = 6. p-AKT: phosphorylated AKT, t-AKT: total AKT. Panel D: ns – non-significant (p > 0.05), Paired Student’s t-test. n = 6. p-PRAS40: phosphorylated PRAS40, t-PRAS40: total PRAS40. Representative images shown on the right.
Figure 7-8. Mechanism of SS-AFSC sEV-induced endothelial cell migration: sEV-induced activation of kinases in the endothelial cells

A,B – Phospho-kinase array for detection of phosphorylated kinases/kinase targets in HUVECs. Cells were incubated with vehicle (PBS) or SS-AFSC sEVs (1x10^{10} particles/ml) for 15 min (panel A) or 3 h (panel B). Results are presented relative to control proteins. Images of the membranes shown in the top right corners in both panels: panel A – Vehicle (top, A053 and B053) and SS-AFSC sEVs (bottom, A054 and B054); panel B – Vehicle (top, A055 and B055) and SS-AFSC sEVs (bottom, A056 and B056). See section 7.3.1 for details about the array membranes.
7.5. Discussion

In this chapter the mechanism of SS-AFSC sEV-induced endothelial cell migration was studied in relation to the sEV cargo mediator and the signalling in the target endothelial cells. Firstly, protein arrays and proteomics were used to identify potential promigratory factors in the SS-AFSC sEV samples. Using these data as guidance, inhibitors of CXCR4 and TLR4 axes as well as antibodies against PTX3 and RTN4B were applied in the Boyden’s Chamber migration assay to study the importance of these pathways. None of them was found to be important for the sEV-promoted migration of endothelial cells. Furthermore, SS-AFSC sEVs failed to activate a panel of kinase pathways in endothelial cells, among which PI3K. Despite that, inhibitors of PI3K pathway reduced SS-AFSC sEV-induced migration of endothelial cells. This indicated that the presence of basal PI3K activity is indispensable for the SS-AFSC sEVs to exert their effects.

In this study, angiogenesis and cytokine arrays identified a number of potential promigratory factors in the SS-AFSC sEV samples, including SDF1 and MIF. SDF1 is a well-known recruiting factor for endogenous progenitor cells\textsuperscript{351} and it induces an angiogenic phenotype of endothelial progenitor cells\textsuperscript{352}. Furthermore, SDF1 was previously shown to provide proangiogenic support to ischaemic hearts decreasing scars and increasing capillary density \textit{in vivo}\textsuperscript{353}. MIF also induces chemotaxis via actions on CXCR4 receptor as shown using primary murine lymphocytes\textsuperscript{349}. However, in the current study pharmacological inhibition of CXCR4 did not affect SS-AFSC sEV-induced HUVEC migration, suggesting that vesicular SDF1 and MIF do not mediate this effect. Interestingly, SDF1 and MIF were only detected using protein arrays while their levels in the sEV samples in the proteomics analysis were below the threshold of detection. This discrepancy between the protein arrays and proteomics may be due to a lack of specificity of the antibodies immobilised to the array membranes. Alternatively, since the levels of all proteins detected using the cytokine array were low, it is possible that the true SDF1 and MIF content of the sEV is negligible and hence they were not detected by proteomics. Taken together, these findings support the notion that
SDF1 and MIF are not mediating the effects of the SS-AFSC sEVs on endothelial cells.

PTX3 was found to be relatively abundant in the sEV samples by both methods used here, and it showed an enrichment in the sEV isolates compared to the starting conditioned medium. Furthermore, PTX3 has previously been shown to induce migration of pancreatic cancer cells\textsuperscript{350}. Therefore, it appeared to be a promising candidate for mediating the SS-AFSC sEV-induced migration of endothelial cells. However, inhibition of the PTX3 with the use of an antibody did not have an impact on the sEV-promoted endothelial cell migration. Further studies will be required to definitively establish whether PTX3 has a role in more complex angiogenic processes, since previous reports were conflicting in that respect showing both proangiogenic\textsuperscript{354} and antiangiogenic\textsuperscript{355} effects of PTX3. Moreover, the mechanism by which PTX3 affects angiogenic processes is currently unclear and it is not known whether it can affect endothelial cell migration.

The lack of effect of the selective TLR4 inhibitor TAK-242 on SS-AFSC sEV-induced endothelial cell migration ruled out any involvement of abundant TLR4 ligands present in the samples, such as BGN\textsuperscript{347}. Importantly, since sEVs were not isolated in a sterile manner, this experiment also eliminated the possibility of contaminant endotoxin-driven migration\textsuperscript{356}.

Finally, RTN4 was among the most abundant proteins in the sEV samples and it showed a marked > 28 times relative enrichment in the sEVs compared to the starting SS-AFSC-conditioned medium by label-free quantification LC-MS/MS. Section 6.4.2 demonstrated that the factor(s) responsible for the effects of the SS-AFSC-conditioned medium are likely to be enriched in the vesicular samples since its promigratory activity eluted with the sEVs and not the soluble protein. An isoform of RTN4, RTN4B (also known as Nogo-B), has been shown to promote chemotaxis of HUVECs \textit{in vitro}\textsuperscript{264} through actions of its N-terminal domain on the NGBR (Nogo-B receptor)\textsuperscript{357}. Interestingly, by use of an antibody against this domain of RTN4B, it was shown here that RTN4B is not involved in the SS-AFSC sEV-induced endothelial cell chemotaxis. Despite being the 9\textsuperscript{th} most abundant protein in the sEV isolates, RTN4 peptide area by label-free LC-MS/MS quantification was < 2\% of the total peptide area of all detected proteins. In the current study, a
migration experiment with 1×10^{10} sEVs/ml approximated to 0.8 µg/ml total protein and taking into account the predicted molecular weight of RTN4B of ~50 kDa (see antibody spreadsheet on https://resources.rndsystems.com/pdfs/datasheets/af6034.pdf), the concentration of RTN4B in a migration experiment was ~0.3 nM. If we consider the fact that this analysis did not distinguish between isoforms of RTN4, RTN4B concentration is likely to have been even lower. Previous reports showed effects of RTN4B on endothelial cell migration only with doses of ≥ 1 nM and this may explain the lack of importance of RTN4B in the current study. However, it should be noted that the LC-MS/MS analysis in the current study was not quantitative and the above calculations are only intended to provide an indication of the predicted RTN4B concentration.

Other proteins in the SS-AFSC sEV isolates can also be responsible for their activity. Previous reports showed that chemoattractants such as VEGF, MCP1 and IL8 are crucial for foetal MSC-conditioned medium-derived migration of endothelial cells^{335} but these factors were not detected at significant levels in the SS-AFSC sEV samples here. In fact, the levels of most proteins detected in the sEV samples by proteomics were < 5% of the estimated total protein amount calculated by label-free LC-MS/MS quantification. Interestingly, type I collagens (COL1A1 and COL1A2) formed > 50% of the total peptide area detected indicative of a remarkably high abundance. It remains unknown whether they are mediating any aspect of HUVEC chemotaxis here but it has been shown before that collagen type I can induce morphological changes in endothelial cells and formation of capillary-like structures^{358}. The rich collagen content of SS-AFSC sEVs may promote migration of endothelial cells similar to the haptotaxis collagens were shown to induce – a directional movement of cells in response to gradients of extracellular matrix components^{341,359}.

Finally, an important different avenue will be the study of miRNAs contained within the SS-AFSC sEVs which have been demonstrated to play important roles in angiogenesis (reviewed in^{67,360}) and may be mediating cardioprotection and cardiac repair conferred by different progenitor cell types^{67,360–362}. Since the isolation method employed in this study yielded highly-pure sEVs, studies on the miRNAs of SS-AFSC sEVs will be very
informative and will avoid common problems of non-sEV miRNA contamination using other sEV isolation techniques\textsuperscript{135,137,363}. It should be noted however, that several arguments have been raised against the possibility of miRNA-mediated effects of MSC sEVs due to reasons such as low miRNA concentration, low proportion of miRNA relative to other ribonucleic acid fragments, and the absence of RNA-induced silencing complex (RISC) proteins in the sEVs which are necessary for mature miRNA function\textsuperscript{115,364}. Nevertheless, an RNA mediated effect of SS-AFSC sEVs cannot be excluded since no studies were performed here to investigate this. Lipid-mediated effects are also possible as discussed elsewhere\textsuperscript{365,366}.

The PI3K pathway is an important player in endothelial cell migration\textsuperscript{339,340} and SS-AFSC sEVs were enriched in PI3K-AKT pathway proteins as analysed by proteomics here. In this chapter it was demonstrated that PI3K signalling in endothelial cells is required for SS-AFSC sEV-induced migration, although sEVs did not directly activate PI3K in HUVECs. This indicates that basal activity of PI3K pathway is necessary for the directional migration of HUVECs towards a gradient of SS-AFSC sEVs. Furthermore, administration of sEVs did not lead to an increase of phosphorylation in any of the kinases/kinase targets in the endothelial cells in the panel used here. Therefore, it appears likely that the sEVs act via other signalling pathways that have not been investigated here. These observations may be due to a more complex signalling owing to the multifaceted actions of the sEVs which contain a variety of biologically active molecules and are expected to deliver numerous proteins and RNAs to the target cells. Therefore, the precise mechanism of SS-AFSC sEV-induced migration of endothelial cells remains to be deciphered in future investigations.

Nitric oxide (NO) was discussed as a hypothetical player in the endothelial-mediated cardioprotection in section 5.5. NO is also central for endothelial cell migration as shown by abrogation of both basal\textsuperscript{367} and VEGF-induced migration\textsuperscript{368} by L-NAME (an eNOS inhibitor) in different bovine endothelial cell populations. Whether NO is important for SS-AFSC sEV-induced promigratory effects remains unknown and it will be intriguing to investigate any NO connection between cardioprotection and endothelial cell migration in future experiments.
Overall, SS-AFSC sEVs carried proteins with promigratory potential. Mechanistically, the chemotactic effects of the sEVs were dependent on PI3K signalling in endothelial cells but not on the abundant SDF1, MIF, PTX3, BGN or RTN4B present in the sEV isolates. Hence, the mechanism of SS-AFSC sEV-promoted endothelial cell migration remains to be deciphered.
Chapter 8. Lipophilic dye labelling as a means of studying the mechanisms of SS-AFSC sEV effects

This chapter has been published as a standalone article (Ref 138):


8.1. Introduction

Understanding sEV uptake into target cells is considered to be essential for deciphering vesicular function and has been intensely studied in relation to the ability of sEVs to deliver proteins, nucleic acids and lipids to recipient cells. The most common approach to visualise vesicular uptake in vitro is by labelling of the sEVs with a fluorescent lipophilic membrane dye comprising of a lipophilic tail (expected to incorporate into the membrane of the sEVs) and a fluorescent moiety (to track the labelled vesicles, e.g. using microscopy). PKH26, PKH67, DiD and CellMask are among the most commonly used fluorescent dyes to label sEVs. Both blood plasma- and AFSC-derived sEVs have been shown to be internalised into target cells by means of lipophilic dye labelling experiments indicating that vesicles may exert their effects through cargo delivery in recipient cells. However, virtually all of the lipophilic dyes will readily incorporate into any lipid-rich structure without being specific to the vesicular membrane. Frequently neglected are the facts that these dyes are widely used to label lipoproteins and they can even bind to proteins. As discussed in the preceding chapters, co-isolation of lipoproteins and proteins is common for EVs isolated from conditioned cell culture medium supplemented with blood products and contaminating soluble protein can also be observed in serum-free conditions. Therefore, it is vital to assess the reliability of this dye labelling method for tracking cellular sEV internalisation.
In this chapter blood plasma and conditioned cell culture medium were used to investigate in detail the validity of the lipophilic dye labelling of sEVs and the contribution of contaminant lipoproteins and soluble proteins to carryover of lipophilic dye into acceptor cells. SEC of rat blood plasma and SS-AFSC-conditioned cell culture medium was employed to obtain vesicle-rich and vesicle-poor fractions. Thereby, it was possible to compare different eluent fractions of the same sample to provide definitive conclusions. Internalisation was studied in endothelial cells with high uptake competency and adult cardiomyocytes which have a reduced propensity for membrane recycling\textsuperscript{64,380}. An additional, alternative approach of directly staining serum and pure albumin samples was also taken to specifically investigate lipoprotein and soluble protein contribution to lipophilic dye retention and transfer to recipient cells.

### 8.2. Aims

The aims of this chapter were to:

1. Use lipophilic membrane dyes to label sEV-rich and sEV-poor SEC fractions from blood plasma to investigate the possibility that contaminants produce artefacts in sEV uptake studies
2. Use lipophilic membrane dyes to label sEV-rich and sEV-poor SEC fractions from SS-AFSC-conditioned medium to investigate the possibility that contaminants produce artefacts in sEV uptake studies
3. Use lipophilic membrane dyes to label albumin samples and ultracentrifugation fractions from serum to investigate the possibility that contaminants produce artefacts in sEV uptake studies
8.3. Methods

8.3.1. Materials

In addition to the other reagents, Exosome-depleted FBS was purchased from ThermoFisher and in-house made EV-depleted FBS was prepared by ultracentrifugation of commercial FBS for 18 h at 100,000 g, 4°C (polycarbonate tubes, 355630, Beckman Coulter; MLA-55 rotor, Optima MAX-XP, Beckman Coulter).

8.3.2. sEV isolation

For SEC of blood plasma, 0.5 ml rat plasma was loaded on a qEVoriginal column (iZON Science) for fractionation. 1.0 – 17.0 ml eluent fractions were collected and analysed.

For sEV isolation from SS-AFSC-conditioned medium, some minor modifications to section 2.2.2.1. were made to adapt the protocols for the aims of this chapter (i.e. serum-supplementation). SS-AFSCs (1x T225 flask, 30 ml culture medium) were washed three times with PBS to remove any residual FBS, and serum-free or Exosome-depleted FBS-supplemented (ThermoFisher) medium was added for 24 h. After the incubation period, centrifugations were performed as explained in section 2.2.2.1., conditioned medium was concentrated using Viviaspin-20 (100 kDa cut-off, polyethersulfone membrane, Sartorius) and processed for sEV isolation by SEC. SEC fractions 3.0 – 18.0 ml were collected and analysed.

Protein, particle and tetraspanin content were normalised to volumes as indicated in the figure legends.

8.3.3. Nanoparticle tracking analysis (NTA)

In addition to the previously explained NTA methods, particle concentration was also measured in FBS, Exosome-depleted FBS (ThermoFisher) and in-house prepared EV-depleted FBS. Samples were diluted and processed as explained in section 2.3.1.
8.3.4. Protein content

Protein content in plasma SEC fractions was estimated by standard BCA assays. Sample aliquots were diluted to 10 µl with PBS and mixed with 190 µl 49 : 1 bicinchoninic acid : copper sulphate solution on a 96-well plate. The plates were incubated for 30 min at 37°C and absorbance was measured at 562 nm on a FLUOstar plate reader (BMG Labtech). Protein concentration was calculated using BSA standards and a 4-parameter logistic curve.

Protein content in SS-AFSC-conditioned medium SEC fractions was estimated by BCA protein assay for low concentrations (ab207002, Abcam) as explained in section 3.3.2.

8.3.5. Dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA)

Antibodies used in this chapter are provided in Table 3-1. Primary antibodies were used at 1 µg/ml in PBS and secondary antibodies at 0.25 µg/ml in PBS.

Table 8-1. Antibodies used for DELFIA studies

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Species / Antibody type</th>
<th>Clone / Cat no</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>1ary</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD9</td>
<td>Mouse IgG1</td>
<td>M-L13</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD81</td>
<td>Mouse IgG1</td>
<td>JS-81</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>HSP70</td>
<td>Mouse IgG1</td>
<td>N27F3-4</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>APOB</td>
<td>Rabbit IgG</td>
<td>H-300</td>
<td>Abcam</td>
</tr>
<tr>
<td>2ary</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse IgG</td>
<td>Goat</td>
<td>ab98691</td>
<td>Abcam</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>Goat</td>
<td>ab97073</td>
<td>Abcam</td>
</tr>
</tbody>
</table>

8.3.6. Triglyceride content

A commercially-available triglyceride assay (Cayman Chemical) was performed according to the manufacturer’s instructions (see section 2.3.7. for further details). 150 µl of the Triglyceride Enzyme Mix was added to 10 µl of each sample. After a 30-min incubation of the reaction at room temperature, absorbance was measured at 530 nm using FLUOstar plate reader (BMG
Labtech). Triglyceride concentration was calculated using triglyceride standards provided by the manufacturer and a 4-parameter logistic curve.

8.3.7. sEV uptake

8.3.7.1. Sample labelling procedure

Uptake of various sEV-rich and sEV-poor SEC fractions in MCECs and ARVCs was studied by means of labelling with CellMask Orange (ThermoFisher), PKH67 (Sigma) or DiD (ThermoFisher) fluorescent dyes.

For CellMask staining of SEC fractions, 50 µl aliquots of each indicated SEC fraction were stained with 7.5 µg/ml CellMask dye (final volume: 500 µl, dilution in PBS). After incubation of the suspensions for 10 min at 37°C, samples were transferred to 0.5 ml ultrafiltration units (Amicon, Merck or Vivaspin, Sartorius) with 100 kDa membrane cut-off. Samples were ultrafiltered at 14,000 g for 5 min for a total of three times with PBS additions to the concentrate before each filtration to achieve thorough washing and removal of unbound dye. Concentrates were collected and used immediately.

For CellMask staining of albumin and serum samples, 20 µl of serum or BSA diluted in PBS were stained as explained above. Where indicated, serum was separated in sEV-rich FBS pellet and sEV-poor FBS supernatant by means of UC. 1 ml of complete FBS was ultracentrifuged for 70 min at 100,000 g, 4°C (polycarbonate tubes, 355630, Beckman Coulter; MLA-55 rotor, Optima MAX-XP, Beckman Coulter). The sEV-rich pellet was then resuspended in 7-8 ml of PBS for washing, and after an additional UC round with the same parameters the pellet was collected and stained.

For PKH67 staining, 100 µl of each SEC fraction (obtained from FBS-supplemented SS-AFSC-conditioned medium) were labelled with 3 µM PKH67 dye diluted in Diluent C (500 µl final volume). After an incubation of 10 min at room temperature, the samples were processed as explained above for CellMask dye.

For DiD staining, 100 µl of each SEC fraction (obtained from FBS-supplemented SS-AFSC-conditioned medium) were labelled with 5 µM
Vybrant DiD dye (500 µl final volume). After an incubation of 10 min at 37°C, the samples were processed as explained above for CellMask dye.

### 8.3.7.2. Cellular uptake of labelled samples

After collection of the stained and washed samples, ~15% of each one was added to MCECs or ARVCs in the respective culture medium (2 ml final volume). Cells were incubated for 3 h at 37°C / 5% CO₂. Cell nuclei were labelled with 5 µg/ml Hoechst 33342 (ThermoFisher). Five images in each group were obtained using a Leica TCS SP5 confocal microscope (laser power: 543 nm – 20%; 405 nm – 9%). Whole image fluorescence intensity was used to estimate dye uptake in MCECs, and membrane fluorescence intensity was used to estimate dye uptake in cardiomyocytes on ImageJ software. Results are presented as arbitrary units (AU).

For the PKH67 staining and uptake, the same procedure for confocal imaging was followed (laser power: 488 nm – 20%; 405 nm – 9%).

For the DiD staining and uptake, the same procedure for confocal imaging was followed (laser power: 633 nm – 20%; 405 nm – 9%).
8.4. Results

8.4.1. Particle, protein, sEV and lipoprotein content of SEC fractions of blood plasma

To investigate the reliability of the lipophilic dye labelling technique to stain sEVs specifically, SEC-fractionated rat blood plasma was firstly used. The presence of lipoproteins and the high soluble protein concentration were used to estimate dye carryover by contaminants. In accordance with the characterisation study of the plasma SEC presented in chapter 3, the peak protein elution was observed at 10.0 ml and an increasing number of particles detected by NTA without an obvious early peak was seen (Figure 8-1A). To reduce the levels of most of the APOB+ large lipoproteins (e.g. chylomicrons, VLDL, IDL\textsuperscript{159}), rats were fasted overnight as suggested previously\textsuperscript{140}. Negligible effects on the total protein content and its elution profile were observed upon fasting, while particle levels in early SEC fractions (< 5.5 ml) were lower in fasted animals (Figure 8-1A-D).

CD9+/CD81+/HSP70+ sEVs eluted with a peak at 5.5-6.0 ml, regardless of the fasting state (Figure 8-1E,F). Strikingly, APOB+ lipoproteins co-eluted with the sEVs with a slightly delayed peak at 6.0-7.0 ml (Figure 8-1E,F). TEM images confirmed the presence of sEV-like structures and abundant spherical particles resembling lipoproteins in the sEV-rich fractions (Figure 8-1G). Triglycerides, mostly found in VLDL and chylomicrons\textsuperscript{252}, were markedly lower in the early SEC fractions of the plasma obtained from fasted rats (Figure 8-1H).

Overall, despite the separation of tetraspanin-positive sEVs in the early SEC fractions from the bulk of the soluble protein in the late SEC fractions, there was a prominent presence of APOB signal (i.e. lipoproteins) in the early sEV-rich SEC fractions.
*Legend on the next page*
Figure 8-1. Characterisation of SEC-fractionated rat blood plasma (see the previous page)

A,B – Concentration of particles and protein of SEC fractions of rat blood plasma collected from non-fasted (A) or fasted (B) animals. C,D – Detail of particle (C) and protein (D) concentration in early SEC fractions (4.0 ml – 5.5 ml) from panel A and panel B. E,F – sEV-specific markers CD9, CD81 and HSP70, and lipoprotein marker APOB in SEC fractions obtained from non-fasted (E) or fasted (F) rats. AU – arbitrary units. G – Electron microscopy image demonstrating the presence of vesicular structures (arrows) and lipoprotein-like species (arrowheads) in SEC fraction 5.5 ml obtained from non-fasted animals. Scale: 200 nm. H – Triglyceride concentration in SEC fractions 4.0 ml – 5.5 ml obtained from plasma of non-fasted or fasted animals.

8.4.2. Interference of contaminants with lipophilic dye labelling of blood plasma sEVs

To compare the retention of the lipophilic dyes by sEV-rich and sEV-poor fractions, early and late plasma SEC fractions were stained in a similar manner and incubated with endothelial cells (MCECs) or cardiomyocytes (ARVCs).

Dye uptake was observed with the stained sEV-rich fraction 5.5 ml for both endothelial cells and cardiomyocytes (Figure 8-2A-D). Unexpectedly, fractions 4.5 ml (only for endothelial cells) and 5.0 ml, containing low concentration of protein and low levels of sEV markers but high triglycerides, showed a comparable uptake to the sEV-rich fraction 5.5 ml (Figure 8-2A-D). Furthermore, vesicle-poor but protein-enriched fraction 8.0 ml showed an astounding ~10-fold higher (for endothelial cells) dye transfer in comparison to sEV-rich fraction 5.5 ml (Figure 8-2A-D). Intriguingly, the observed reduction of triglyceride content in fractions 4.5 ml and 5.0 ml after overnight fasting resulted in a decrease of the retention of the dye and its transfer to both endothelial cells and cardiomyocytes (Figure 8-2A-D). As expected, the free dye was completely cleared from the samples by the filtration procedure as shown by the negative control vehicle (PBS) stained sample (Figure 8-2A,C).

These findings suggest that non-sEV components may bind the commonly used lipophilic dyes and subsequently obscure any genuine uptake of labelled sEVs.
Legend on the next page
8.4.3. Interference of contaminants with lipophilic dye labelling of SS-AFSC sEVs

Next, SEC-fractionated SS-AFSC-conditioned medium was labelled with lipophilic dyes to assess the applicability of this method for studies with foetal sEVs, but also to evaluate the potential of contaminant artefacts in comparison to plasma samples in section 8.4.2. Both serum-free and serum-supplemented (with EV-depleted FBS) media were used to ensure evaluation of dye binding to both lipoproteins and soluble protein.

As shown in chapter 4, SS-AFSC sEVs eluted with an early CD81 peak (fractions 4.0 ml – 4.5 ml; Figure 8-3A,B). Expectedly, sEVs isolated from serum-supplemented conditions were in higher numbers and had more protein content which is likely to be due to SS-AFSCs proliferation in serum-supplemented conditions and therefore greater number of cells (Figure 8-3A-C). Similar to section 8.4.2., the highest fluorescence uptake was observed with fractions virtually devoid of sEVs but rich in protein and possibly lipoproteins for the serum-supplemented condition (i.e. fraction 8.0 ml; Figure 8-3D,E). Since serum-free fraction 8.0 ml contains no sEVs but also no lipoproteins, the finding that it can transfer fluorescent stain to recipient cells is of great importance as it demonstrates that the dye can readily bind to soluble protein.

Analogous to animal fasting, serum deprivation led to a marked decrease of staining in early fractions 4.5 ml and 5.5 ml (Figure 8-3D). Moreover, dye transfer did not correlate with CD81 marker levels, as serum-supplemented fraction 5.5 ml contained similar CD81 levels but showed considerably higher
fluorescence carryover compared to serum-free fraction 5.5 ml (Figure 8-3B,D).

To draw more generalised conclusions, two other common lipophilic dyes were used, *i.e.* PKH67 and DiD. In support of the findings above, sEV-poor fraction 8.0 ml transferred noticeably more fluorescent dye than sEV-rich fraction 4.5 ml confirming the general non-specificity of the lipophilic membrane dyes (Figure 8-4A,B).

Overall, no correlation between lipophilic dye uptake and sEV marker signal was found for SEC fractions of SS-AFSC-conditioned medium. These findings show that commonly used fluorescence dyes are likely retained by various components of the cultured medium including non-EV, cell-derived protein.
Figure 8-3. Characterisation of SEC fractions from SS-AFSC-conditioned medium and their endothelial cell uptake

A – Amount of protein and particles in SEC fractions of serum-supplemented (FBS) or serum-free (SF) medium conditioned by SS-AFSCs. B – Amount of protein and CD81 signal in SEC fractions of serum-supplemented (FBS) or serum-free (SF) medium conditioned by SS-AFSCs. C – Amount of protein in SEC fractions 3.0 ml – 5.5 ml from panel A and panel B. D,E – sEV-rich or sEV-poor SEC fractions collected from serum-supplemented (FBS) or serum-free (SF) medium conditioned by SS-AFSCs were stained with CellMask Orange fluorescent
membrane dye. MCECs were incubated with the labelled samples and fluorescence uptake investigated. AU – arbitrary units. n = 5 images acquired in a single representative experiment shown on panel D. Representative images from serum-supplemented condition are shown on panel E. Scale: 50 µm.

Figure 8-4. Impact of the choice of lipophilic dye on the uptake of SEC fractions from SS-AFSC-conditioned medium in endothelial cells

A,B – Representative images of the uptake of PKH67 (A)- or DiD (B)-labelled SS-AFSC-conditioned medium (serum-supplemented) SEC fractions into MCECs. Right panels are zoomed in sections of the left panels. Scales: 50 µm (PKH67: left and right panels and DiD: left panels); 20 µm (DiD: right panels). Hoechst staining was used to label the nuclei in both panels.
8.4.4. Lipophilic dye labelling of serum and pure protein samples as a means of investigating contaminant artefacts in uptake experiments

To confirm the findings obtained above, another approach was taken by using samples of serum (i.e. FBS) or purified protein (i.e. BSA). Complete FBS was used in this section together with a commercially-available Exosome-depleted FBS and in-house prepared (by ultracentrifugation) EV-depleted FBS. As expected, both EV-depleted sera were found to contain decreased number of particles and protein concentration in comparison to the complete serum (Figure 8-5A,B).

Serum aliquots were stained with fluorescent lipophilic dye in a similar manner to the SEC samples of blood plasma and conditioned medium in sections 8.4.2. and 8.4.3. FBS retained the dye and transferred it to the recipient endothelial cells, while depletion of EVs from the FBS reduced the fluorescence transfer by ~15% for the in-house prepared EV-depleted FBS and ~28% for the commercially available EV-depleted FBS (Figure 8-5C,D). This reduction correlated with both particle and protein content (Figure 8-5A-D). Since it was shown above that the lipophilic dyes may also bind to soluble protein, the protein-dye binding was directly studied using a pure protein sample of albumin (BSA) at a protein concentration of 33 mg/ml, similar to the one measured in the complete serum (Figure 8-5B). Unexpectedly, BSA formed complexes with the fluorescent dye which were retained after filtration and transferred to the recipient endothelial cells (Figure 8-5C,D). This fluorescent dye transfer was ~46% of the fluorescence observed with complete serum. Similar to the experiments with endothelial cells, labelled sera and BSA samples were all able to carry over dye to cardiomyocytes (Figure 8-5E,F).

To directly address the question of whether serum EVs are the main contributors of dye transfer, ultracentrifugation of complete serum was used to separate it into vesicle-rich pellet and vesicle-poor supernatant. The majority of serum particles detected by NTA were not pelleted by the ultracentrifugation step (Figure 8-6A), which was expected since plasma nanoparticles have previously been shown to be mostly non-sEV structures. Almost all of the protein was retained in the serum supernatant while protein content in the
serum pellet was low (Figure 8-6B). As observed above, complete serum retained and transferred the lipophilic dye efficiently (Figure 8-6C,D). Strikingly, only ~1% of the fluorescence transfer with complete serum was precipitated in the serum pellet, while most of it was found to be due to supernatant retention of the dye (Figure 8-6C,D).

In summary, these findings indicate that EVs are likely to contribute only marginally to lipophilic dye transfer to acceptor cells. Most of the fluorescence transfer was found to be associated with EV-poor samples and therefore various contaminants in the sEV samples may mask any existing vesicular uptake.
A  
Serum particle size distribution  

B  
Serum protein concentration  

C  
Endothelial cell uptake of labelled FBS  

D  
FBS  
FBS + O/N UC  
Exo-free FBS  
BSA  

E  
Cardiomyocyte uptake of labelled FBS  

F  

*Legend on the next page*
**Figure 8-5. Uptake of labelled serum and pure protein samples in endothelial cells and cardiomyocytes (see the previous page)**

A,B – Size-distribution profile and concentration of particles (A) and protein content (B) in complete serum (FBS), in-house prepared EV-depleted serum (FBS + O/N UC) and commercially-available Exosome-depleted FBS (Exo-free FBS). C-F – FBS, FBS + O/N UC, Exo-free FBS and albumin (BSA, 33 mg/ml) samples were stained with CellMask Orange fluorescent membrane dye. MCECs (C,D) or ARVCs (E,F) were incubated with the labelled samples and fluorescence uptake investigated. Non-stained – background cell fluorescence. AU – arbitrary units. n = 5 images acquired in a single representative experiment in panels C and E. D,F – Representative images of panels C and E. Uptake of dye-labelled complete serum in cardiomyocytes is shown on panel E. Scales: 50 μm (panel D), 20 μm (panel F).
Figure 8-6. Uptake of serum and UC-derived serum fractions in endothelial cells

A,B – Particle size-distribution profile (A) and protein content (B) in complete serum (FBS), UC-derived serum pellet (FBS pellet) and UC-derived serum supernatant (FBS supernatant).

C,D – FBS, FBS pellet and FBS supernatant were stained with CellMask Orange fluorescent membrane dye. MCECs were incubated with the labelled samples and fluorescence uptake investigated. Dye-only – vehicle (PBS) stained with the lipophilic dye to control for non-specific dye carryover. Non-stained – background cell fluorescence. AU – arbitrary units. n = 5 images acquired in a single representative experiment in panel C. D – Representative images of panel C. Scale: 50 µm. Inserts at the bottom-right are with increased intensity compared to their original images. Note the considerably higher fluorescence in the FBS pellet group compared to the Dye-only group.
8.5. Discussion

In this chapter the reliability of fluorescent lipophilic dye staining as a commonly used technique for sEV labelling and tracking of sEV uptake in acceptor cells was evaluated. Using SEC fractions of blood plasma and serum-free or serum-supplemented SS-AFSC-conditioned medium, it was shown that lipophilic dye tagging is largely non-specific and dye molecules readily bind to proteins and lipoproteins co-isolated with the sEVs. Staining of complete serum and its sEV-rich and sEV-poor fractions, together with labelling of pure protein samples further confirmed these findings, demonstrating that non-sEV proteins and lipoproteins contribute to a large proportion of the observed lipophilic dye carryover. Thus, discrimination between uptake of labelled sEVs and co-purified contaminants in subsequent experimentation is virtually impossible.

In line with the findings presented in chapter 3, APOB+ lipoproteins were co-purified with sEVs when using SEC of blood plasma. SEC fractionation allowed for specific investigation of samples derived from a single source which are either rich in sEVs or poor in sEVs but rich in soluble proteins and lipoproteins. Interestingly, lower particle numbers, triglyceride content and APOB signal in early SEC fractions 4.5 ml and 5.0 ml were observed in samples collected from overnight-fasted rats, likely due to a reduction in chylomicrons and VLDL. Precise quantification of lipoprotein content changes is beyond the scope of this study and it was reported elsewhere but it seems plausible that the reduction of lipoproteins and triglycerides in these early fractions collected from fasted rats was the reason for the reduced fluorescence uptake with these fractions. This is not surprising as multiple previous reports investigated cellular uptake of various lipoprotein particles labelled with lipophilic dyes including VLDL, LDL and HDL. The contribution of lipoproteins to the non-specific lipophilic dye transfer is also seen in the experiments using serum labelling. Direct labelling of both commercial and in-house prepared EV-depleted serum showed considerable fluorescence retention which accounted for 70-85% of the complete FBS fluorescence carryover. Furthermore, applying a standard ultracentrifugation protocol to isolate sEVs from serum demonstrated that virtually none of the
lipophilic stain transfer of the complete FBS precipitates in the FBS sEV-rich pellet. Taken together, these are clear indications that sEV contribution to dye transfer may be small and is frequently masked by the presence of other particulate material in the samples. Notably, this is of extreme importance for experiments with serum-supplemented conditioned medium. As discussed in section 1.6.2., starting volumes of conditioned medium in these studies may sometimes be > 100 ml\textsuperscript{109}. Supplementation of medium even with 1% FBS will lead to concentration of the lipoproteins from > 1 ml serum which is expected to confound subsequent sEV uptake studies.

In fact, in the experiments with serum samples and serum fractions, there was an apparent correlation between total protein content and subsequently observed fluorescence transfer. Therefore, it seemed possible that fluorescent dyes can also be bound by soluble protein present in the sEV isolates. This was clearly evident in labelled samples containing pure protein (\textit{i.e.} BSA) and in labelled sEV-poor SEC fractions collected from serum-free SS-AFSC-conditioned medium.

BSA readily bound the lipophilic dye and transferred significant amounts to both endothelial cells and cardiomyocytes. This was surprising since the processing of the samples involved 100-kDa membrane ultrafiltration step in combination with a few wash cycles which should theoretically have eliminated the 66 kDa\textsuperscript{321} BSA from the resulting concentrate. This observation may be explained with aggregation of the BSA molecules\textsuperscript{381} and/or the inability of the ultrafiltration unit to eliminate completely the dye-bound BSA molecules. Importantly, free dye carryover was excluded as a possibility here, since filtration step completely eliminated the non-bound dye in the samples.

The main purpose of this chapter was to evaluate the validity of the lipophilic dye staining method and to investigate the internalisation of sEVs in endothelial cells and cardiomyocytes as a potential mechanism of action of the SS-AFSC sEVs. Surprisingly, experiments using SEC fractions from SS-AFSC-conditioned medium showed striking similarities to the plasma SEC fraction labelling. Dye carryover to recipient cells was most pronounced with sEV-poor SEC fraction 8.0 ml which was rich in proteins and likely lipoproteins in serum-supplemented conditions. Strikingly, even in serum-free environment, fraction 8.0 ml transferred > 50 times more fluorescent signal to
recipient cells than sEV-rich fraction 5.5 ml. Fraction 8.0 ml contained negligible number of particles and very low sEV marker signal. This is a clear indication that lipophilic dyes are prone to interact with many proteins, not only high-abundance or high-binding ones such as albumin. These findings definitively show that even in serum-free conditions, non-sEV components contribute to dye transfer to a much greater extent than the sEVs in a vesicular isolate.

Protein- and lipoprotein-(for serum-containing samples) rich fraction 8.0 ml demonstrated the highest fluorescence transfer in all plasma samples, serum-supplemented or serum-free samples. Since both lipoproteins and soluble protein were shown to contribute to dye carryover, it was virtually impossible to distinguish between vesicular transfer of fluorescence or contaminant-associated artefacts. For instance, the richest sEV fractions from plasma samples consisted of a complex mixture of sEVs, lipoproteins and soluble proteins and it would be difficult to identify the origin of any transferred fluorescence. Furthermore, a closer look at fraction 5.5 ml of SS-AFSC-conditioned medium showed a discrepancy between sEV content and dye uptake: serum supplementation of the medium resulted in markedly increased fluorescence transfer in comparison to serum-free conditions, regardless of the similar sEV marker content in both. This was likely due to protein- and/or lipoprotein-dye binding in serum-supplemented isolates. Notably, although less likely, the possibility for inherent differences in the sEVs due to serum deprivation that may affect subsequent uptake cannot be excluded. Such differences have been reported for the protein cargo of the vesicles derived from other cell types but it is not known whether sEV lipid composition can be altered in serum-free environment and whether this could affect lipophilic dye interactions with the vesicular membrane.

The main dye used for sample labelling here was CellMask Orange as it is suggested to provide more uniform staining and improved signal with an easier application protocol (see also CellMask brochure at https://assets.thermofisher.com/TFS-Assets/LSG/manuals/CellMask_Plasma_Membrane_Stains_Pl.pdf). Nevertheless, two additional lipophilic dyes – PKH67 and DiD – were also used which showed similar results and confirmed that the observations were not due
to a specific characteristic of the CellMask stain. It is interesting to note that these dyes are intended for cell-tracking by plasma membrane staining and they are not designed as specific EV-labelling agents. In fact, the brochure for the PKH67 dye specifically instructs the operator to use BSA as a dye scavenger after cell staining\textsuperscript{379}, thus exploiting the high-binding abilities of the albumin molecules to remove the excess dye. Seemingly, this remained unnoticed by virtually all previously published experiments using lipophilic dyes to label vesicle samples.

It was further demonstrated here that similar results are obtained with endothelial cells and adult cardiomyocytes. In the latter case, cardiomyocyte membranes are readily labelled with the fluorescent dye-labelled samples, regardless of the sample sEV content. Considering this characteristic labelling of the membrane, it is possible that dissociation of the dye, previously bound to vesicles, proteins or lipoproteins, causes staining of the cardiomyocyte membranes. Such a dye leakage has been reported before\textsuperscript{384} and may account for these observations. Regardless of the mechanism of dye carryover, the similarity of the cardiomyocyte uptake with endothelial cell uptake and the transfer of fluorescently-labelled BSA and EV-depleted sera to cardiomyocytes further supports the non-specific nature of this labelling approach.

Our dye-only sample controls ensured that the dye was not carried over in micelles or other aggregates to the recipient cells, but it was bound to cell- or plasma-derived products. Despite the non-specificity of the dye binding, in some circumstances where purity can be ensured, e.g. in serum-free environment, lipophilic dye labelling may be applicable. In such a case losses and processing time could be reduced by labelling the starting conditioned medium and purifying it on SEC for removal of the free dye and dye-protein complexes and isolation of the labelled pure sEVs. However, it has to be considered that since lipophilic dyes can readily bind to protein molecules, it is possible that even in completely pure sEV populations dyes adhere to the outer surface of the vesicles or vesicular membrane proteins and are subsequently freed to cause false positives. As explained above, such dye leakage has been reported previously\textsuperscript{384}. Inclusion of appropriate sEV-free and protein-only
controls would be necessary to control for the aforementioned confounders in future experiments involving lipophilic dye labelling.

Non-specificity of the fluorescent lipophilic dyes has also been reported by others. One study showed that PKH67 staining of EV-depleted medium leads to a dye transfer 2.5 times higher than the one observed with staining of the isolated EVs\textsuperscript{260}. The authors showed that genetic labelling by means of transfection with fluorescent protein targeted to membranes is much more specific for tracking the fate of the EVs\textsuperscript{260}. This technique has also been employed by others and may be a viable alternative to the lipophilic dye staining\textsuperscript{260,307,385,386}. More complex genetic approaches used Cre recombinase transfection in EV-producer cells and \textit{loxP}-regulated expression of a reporter protein in EV-recipient cells\textsuperscript{387}. However, these approaches are only applicable to cultured cells and are not useful for biological fluid sEV isolates.

In this chapter it was clearly shown that fluorescent lipophilic dyes can bind to non-sEV components of the samples of interest, questioning their validity as an experimental approach to label sEVs specifically. Unfortunately, this method could not be applied with confidence to studying the mechanism of SS-AFSC sEV uptake. Furthermore, caution should be exerted when interpreting the findings of any experiment involving lipophilic dye labelling of vesicles. Our findings question the reliability of previous reports for uptake of plasma- and cell culture medium-derived vesicles and show the need for developing more specific techniques to track vesicle internalisation in target cells.
Chapter 9. Summary and outlook

9.1. Summary of findings

MI remains a major contributing factor to increased morbidity and mortality. Discovering therapies that protect the heart, improve its blood supply and repair the myocardial damage to combat IRI is of great necessity. The use of foetal stem cell sEVs may provide a new opportunity in this respect and deliver the long-sought clinical cardioprotective intervention. However, prior to clinical application, rigorous studies of the sEV isolation protocols and the obtained samples must be performed to ensure smooth translation into clinic.

SEC has found its relatively novel application in the sEV field as it provides flexibility, better sample purity and preserved vesicular morphology and biological potency. In this thesis it was firstly demonstrated that contaminants in blood plasma sEV isolates, such as abundant APOB+ lipoproteins and soluble protein, are in excess and interfere with blood derived sEV purification using SEC. This is of the uttermost importance when using conditioned medium supplemented with blood products (e.g. serum).

Hence, SEC was used to isolate sEVs from serum-free SS-AFSC-conditioned medium and the harvests were characterised in detail. The isolated vesicles were found to be of a very high purity according to published guidelines, while some contaminants were detected in the isolates including trace bovine proteins and ribosomal/nucleosomal proteins. This was likely due to the remnants of prior serum incubation and to the nature of SEC isolation, respectively.

SS-AFSC sEV activity on cardioprotection was then studied. sEVs were shown to possess cardioprotective properties in vivo, but not in vitro – an indication for an indirect cardioprotective actions via intermediate effects on other cells or organs. An attempt was made to investigate the involvement of the stable endothelial cell secretome in this process, but no specific cardioprotective mechanism was identified.

The effects of SS-AFSC sEVs on angiogenesis in vitro were also studied and shown to be confined to sEV-induced endothelial cell migration, with minor
or no effects on endothelial cell proliferation and tube formation. Intriguingly, sEV application was sufficient to recapitulate the SS-AFSC-conditioned medium-induced migration of endothelial cells. sEV-promoted endothelial cell migration was mediated via the PI3K pathway in recipient endothelial cells. The possible involvement of some abundant sEV proteins in this activity was studied but the mediator of endothelial cell migration in the sEV cargo remained elusive. The rest of the rich sEV cargo identified by proteomic analysis remains to be studied in further detail.

Finally, a commonly used approach for tracking the uptake of the sEVs in target cells by use of fluorescence lipophilic dyes was evaluated by a systematic comparison of labelled blood plasma- and SS-AFSC-derived SEC eluents, serum fractions and pure protein samples. It was clearly demonstrated that the lipophilic dye tagging is not specific to the sEVs in a vesicular isolate and the fluorescence is more likely to be carried over to the recipient cells by contaminants in the sEV samples such as lipoproteins and soluble non-sEV protein.

### 9.2. Outlook and further studies

With regard to the isolation of sEVs from blood plasma or serum-supplemented conditioned medium by SEC, it may be possible to resolve the issue of lipoprotein contamination by removing lipoproteins following the isolation procedure. Purification methods have recently been studied including immunoaffinity separation by antibody-coated beads or a more sophisticated coupling of microfluidics and acoustics. However, the former method may not be able to completely clear all lipoproteins from the samples and at the same time it may remove some EVs non-specifically. Moreover, the latter approach requires production of specific devices that may not be readily-available and easily-applied, and is unlikely to achieve high yields.

SEC isolation used here appears to hold a lot of promise for isolation not only of SS-AFSC sEVs but also of other MSC sEVs. Further purification of the samples and removal of the residual bovine proteins will be required to make the isolates clinically applicable in the future. Clearance of bovine proteins could be achieved via serum-devoid culture from the point of cell isolation. This
has been done previously for CPC-derived sEVs\textsuperscript{203} and the most promising way may be to use defined medium, xenogeneic-free environment and Good Manufacturing Practice (GMP)-compatible reagents.

Removal of ribosomal or nucleosomal proteins from the SS-AFSC sEV samples may also be beneficial. This, however, will require additional steps such as density-based purification\textsuperscript{115}. Since the proteomic analysis performed here was mostly qualitative and only relative abundances of proteins were recorded, it is not clear whether the presence of ribosomal proteins or histones is biologically significant and impacting the subsequent functional activity. Quantitative proteomic analysis should be employed in the future to provide better estimates of the exact amount of these proteins in the sEV isolates.

As part of the medicinal category of “biological medicines” or “biologics”, in order for a successful translation of the sEVs into clinic, the requirements that have to be met include accurate quantification of the active substances and understanding of the mechanistic basis of the sEV-mediated effects, but also characterisation of the non-active components in the isolates\textsuperscript{390}. Therefore, any impurities in the sEV isolates, even if they are inert at a given sEV dose, must be thoroughly characterised prior to a therapeutic application of the sEVs. Attention has to be given to any false positive signals due to the presence of contaminating factors or potential toxic side effects of the impurities, including unwanted cell reprogramming by foreign genetic elements and tumorigenesis.

SS-AFSC sEVs were shown here to possess cardioprotective potential but the mechanism behind these effects was not deciphered. The involvement of endothelial cells should be further studied using additional models, e.g. in co-cultures of endothelial cells and primary cardiomyocytes, to account for effects of the non-stable endothelial secretome. The involvement of other biological signalling mechanisms should also be considered such as neural pathways\textsuperscript{173} and immune response\textsuperscript{67} which were previously shown to be intermediary cardioprotective signals.

The mechanism of SS-AFSC sEV-promoted endothelial cell migration requires further investigation. The proteomic studies presented here may serve as further guidance, but miRNA and lipid sEV cargo should also be considered. Additionally, the lack of proangiogenic effects seen in the \textit{in vitro
tube formation assay should also be confirmed *in vivo* to conclusively determine whether SS-AFSC sEVs have any influence on formation of new blood vessels post myocardial ischaemia.

Generally, sEVs are considered to be a safer alternative to using their source stem cells since they are not expected to generate tumours. However, even if sEVs are not carriers of DNA as proposed recently115, it has been shown that miRNAs can be tumorigenic391. Moreover, some of the putative tumorigenic miRNAs, such as miRNA-21, have been detected in MSCs sEVs and even proposed to play roles in their cardiac effects362. In light of this, DNA-free reprogramming induced by sEVs should be carefully examined and ruled out to ensure clinical safety of the administered sEVs, especially in cases where pluripotent stem cells are used as sEV producers.

sEVs have a diverse repertoire of activities which also includes some potential multitarget detrimental effects. The ability of the MSC sEVs to promote proliferation of fibroblasts392,393 should be considered in the MI setting as profibrotic effects in the heart are highly undesirable. Furthermore, procoagulant activities due to the presence of coagulation factors in the sEV cargo or the well-known exposure of phosphatidylserine on the sEV surface118,394 should also be taken into account in any future safety studies.

Donor differences should also be considered for future clinical application of SS-AFSC sEVs. These have not been investigated in the current study but previous reports demonstrated that significant differences exist between donor MSCs in the levels of proangiogenic factors in their secretomes and also in the functional potential of these secretomes to drive angiogenesis *in vitro*335.

Nevertheless, sEVs hold great promise and have a number of advantages as a cell-free therapy: they can be isolated in large quantities and in a sterile manner, stored and provided as an off-the-shelf therapy; sEVs may be engineered to transfer a desired cargo or to target a specific organ or cell type; their size is unlikely to cause vascular obstruction, and unlike their parental cells, there are currently no reports for tumorigenic effects of MSC sEVs, ectopic differentiation into unwanted cell types or cell death-associated acute inflammatory responses *in vivo*.

Overall, this thesis sheds light on the cardiovascular effects of sEVs obtained from foetal MSCs. Upon addressing the limitations listed above, this
research may be a basis for future development of cardioprotective and proangiogenic therapies for patients who have suffered a myocardial infarction.
Chapter 10. References


51. Lecour S. 2009. Activation of the protective Survivor Activating Factor


186. Strauer BE, Steinhoff G. 2011. 10 years of intracoronary and intramyocardial bone marrow stem cell therapy of the heart: From the methodological origin to...


immunoassays. Anal Bioanal Chem. 400(9): 2847–64.


379. Sigma. PKH67 Fluorescent Cell Linker Kits For General Cell Membrane Labeling.


