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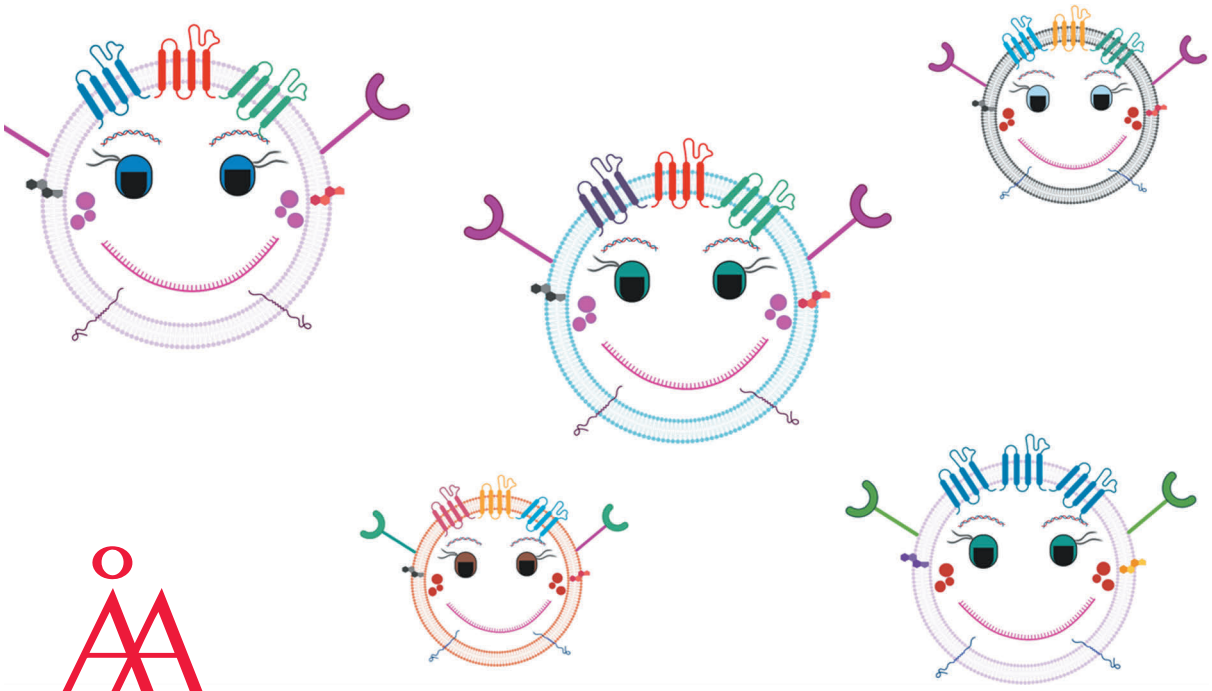

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The Role of Exosomal Vimentin in Mediating Wound Healing

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Åbo, Finland, 2022

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ISBN 978-952-12-4237-3 (printed)
ISBN 978-952-12-4238-0 (digital)
Painosalama, Turku/Åbo, Finland 2022

To my beloved parents

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Abstract

Despite great advances in tissue engineering and regenerative medicine, impaired wound healing is still a challenging clinical problem. Accumulating evidence demonstrates the ability of extracellular vesicles and specifically, exosomes in regenerative therapy and tissue engineering. Previous studies showed that adipose stem cell-derived exosomes have great potential in accelerating cutaneous wound healing by affecting fibroblast activities. It has been shown that vimentin serves as a coordinator of the healing process. Interestingly, vimentin has been reported to be detectable in exosomes from different cell types which we called exosomal vimentin. Therefore, we hypothesized that vimentin incorporated into the exosomes may contribute to mediating fibroblast activities in wound healing.

During my Ph.D. thesis, we revealed the active and necessary role of exosomal vimentin in promoting wound healing. Our results revealed that exosomal vimentin from adipocyte progenitor cells acts as a promoter of fibroblast proliferation, migration, and ECM secretion. Our results suggested that exosomes can serve as an efficient transportation system to deliver and internalize vimentin into target cells, while vimentin could have an impact on exosome transportation, internalization, and cell communication. Furthermore, our findings revealed that during mechanical stress such as osmotic imbalance, exosomal vimentin can protect fibroblasts against stress and inhibit stress-induced apoptosis. These data suggest that exosomes could be considered either as a stress modifier to restore the osmotic balance or as a conveyer of stress to induce osmotic stress-driven conditions. In conclusion, our *in vitro* and *in vivo* experiments provide evidence that exosomal vimentin shortens the healing time and reduces scar formation.

Abstrakt

Trots stora framsteg inom vävnadsteknik och regenerativ medicin, så är försämrad sårläkning fortfarande ett stort problem inom klinisk medicin. Det finns allt starkare belägg för att extracellulära vesikler, särskilt exosomer, har sårläkande förmåga i samband med regenerativ terapi och vävnadsteknik. Tidigare studier har visat att exosomer som härrör sig från adipocyta stamceller har stort potential i påskyndandet av hudens sårläkning genom deras förmåga att påverka aktiviteten hos fibroblaster. Man har visat att vimentin fungerar som en koordinator för läkningsprocessen. Vi har kunnat påvisa närvaro av vimentin i exosomer från olika celltyper, som vi kallar exosomalt vimentin. Därmed antog vi att vimentin inkorporerat i exosomer kunde bidra till förmedlingen av fibroblastaktiviteten vid sårläkning.

Under mitt doktorsarbete har vi påvisat exosomalt vimentin är nödvändigt för att främja sårläkning. Våra resultat visade att exosomalt vimentin från adipocyt-progenitorceller stimulerar fibroblastproliferation, migration och ECM-sekretion. Våra resultat antydde att exosomer kan fungera som ett effektivt system för att transportera och internalisera vimentin i målceller, medan vimentin i sin tur kan ha en inverkan på exosomtransport, internalisering och cellulär kommunikation. Dessutom visade våra resultat att exosomalt vimentin under mekanisk stress, såsom osmotisk obalans, kan skydda fibroblaster mot stress och hämma stressinducerad apoptos. Dessa data indikerar att exosomer kan betraktas antingen som stressmodifierare för att återställa osmotisk balans eller som en stressbärare för att inducera osmotiska stressdrivna tillstånd. Sammanfattningsvis visar våra experiment, både *in vitro* och *in vivo,* att exosomalt vimentin försnabbar sårläkning på ett signifikant vis och att det även minskar ärrbildning.

List of Publications

Parvanian S*, Yan F*, Su D, Coelho-Rato LS, Venu AP, Yang P, Zou X, Jiu Y, Chen H, Eriksson JE, and Cheng F. (2020), Exosomal vimentin from adipocyte progenitors accelerates wound, *Cytoskeleton Journal*, 77: 399–413

Parvanian S*, Zha H*, Su D, Xi L, Jiu Y, Chen H, Eriksson JE, and Cheng F. (2021), Exosomal vimentin from adipocyte progenitors protects fibroblasts against osmotic stress and inhibits apoptosis to enhance wound healing, International *Journal of Molecular Sciences*, 22: 4678.

Nuopponen M, **Parvanian S**, Eriksson JE, Cheng F, Sheard J, and Kiuru T. (2021), Bioreactor and a method for separating cell-derived products from cultured cells and a nanostructured cellulose product, Filed Patent application.

Other Publications Not Included in the Thesis

Parvanian S, Coelho-Rato LS, and Eriksson JE. (2022), Extracellular vimentin in health and disease, manuscript.

Su D, Tsai H, Xu Z, Yan F, Wu Y, Xiao Y, Liu X, Wu Y, **Parvanian S,** Zhu W, Eriksson JE, Wang D, Zhu H, Chen H, and Cheng F. (2019), Exosomal PD-L1 functions as an immunosuppressant to promote wound healing, *Journal of Extracellular Vesicles*, 9: 1709262.

*These authors contributed equally to this work

Author Contributions

I. The author contributed to designing and performing *in vitro* experiments, analyzing data, and writing the manuscript. Particularly, the author designed and performed HDFs and APCs cultures, extracellular vesicles isolation, purification, and characterization including western blotting, TEM microscopy, and DLS analysis, wound scratch assay, cell proliferation, migration, and transfection assays, ECM production, characterization, and quantification assays. Coelho-Rato LS performed the mass spectrometry experiment for isolated exosomes, analyzed data, and wrote the related parts in the manuscript. Yang P assisted with exosome isolation. Venu AP. assisted with STED microscopy imaging for isolated exosomes. The author together with Cheng F's research group at Sun Yat-Sen University in China designed the *in vivo* experiment. However, performing, analyzing data, and documentation of animal experiments were done by her research group with the contribution of Yan F, Su D, and Zou X. All authors discussed and commented on the manuscript. Revision and proofs for the publication were done by the author assisted by Eriksson JE, Cheng F, and Coelho-Rato LS.

II. The author contributed to designing and performing the experiments, analyzing data, and writing the manuscript. Specifically, the author designed and performed *in vitro* experiments including HDFs and APCs cultures, osmotic stress induction, extracellular vesicles isolation, purification and characterization including western blotting, TEM microscopy, DLS and NTA analysis, exosome labeling, uptake and quantification assays, cell proliferation and apoptosis assays, ECM production, characterization, and quantification assays. The author together with Cheng F's research group at Sun Yat-Sen University in China designed the *in vivo* experiment. However, performing, analyzing data, and documentation of animal experiments including mouse skin injury and treatment, histology, and qPCR were done by her research group with the contribution of Zha H, Su D, and Xi L. All authors discussed and commented on the manuscript. Revision and proofs for the publication were done by the author assisted by Eriksson JE, and Cheng F.

III. Prompted by the challenges in the production of exosomes in publications I and II, the author conceptualized the idea of combining two separate methods including 3D cultures using nanofibrillar cellulose and CELLine Adhere 1000 (CLAD1000) flask to enhance exosome production. The author specifically contributed to designing and performing the experiments including HDFs and APCs 2D and optimization of 3D cultures, extracellular vesicles isolation, purification, and characterization including western blotting, TEM microscopy, and NTA analysis, exosome labeling, uptake and quantification, and cell proliferation assay, analyzing data, and writing the patent report. Nuopponen M, Sheard J, and Kiuru T, assisted in designing experiments and writing the patent report experiment. All authors discussed and commented on the final draft of the patent application. The method described in the patent can be used for the practical and clinical implementation of exosomal vimentin.

Abbreviations

2D: Two-dimensional
3D: Three-dimensional
ADP: Adenosine diphosphate
Alix: Programmed cell death 6 interacting protein
APCs: Adipocyte progenitor cells
APC-Exos: APC-derived exosomes
ASCs: Adipose-derived stem cells
ASC-Exos: ASC-derived exosomes
bFGF: Basic fibroblast growth factor
CD: Cluster of differentiation
CDMs: Cell-derived matrices
CNS: Central nervous system
Dil: Dioctadecyl-3,3,3′,3′- tetramethylindocarbocyanine perchlorate
DLS: Dynamic light scattering
DNA: Deoxyribonucleic acid
DMSO: Dimethyl sulfoxide
ECM: Extracellular matrix
EGF: Epidermal growth factor
EMT: Epithelial-mesenchymal transition
ER: Endoplasmic reticulum
EVs: Extracellular vesicles
ESCRT: Endosomal sorting complexes required for transport
Exo: Exosome
FBS: Fetal bovine serum
FGF: Fibroblast growth factor
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
GFAP: Glial fibrillary acidic protein
H^-: Hypo-osmotic stressed
H^+: Hyper-osmotic stressed
HDFs: Human dermal fibroblasts
HSP70: Heat shock protein 70
HIV: Human immunodeficiency virus
kg: Kilogram
kD: Kilo Dalton
IF: Intermediate filaments
IFN-γ: Interferon Gamma
IL: Interleukin

MMPs: Matrix metalloproteinases
mOsm: Milliosmole
MVBs: Multivesicular bodies
MSCs: Mesenchymal stem cells
MSC-Exos: MSC-derived exosomes
MTT: 3-[4.5-Dimethylthiazol-2-yl]-2.5-diphenyltetrazolium bromide
NADPH: Nicotinamide adenine dinucleotide phosphate
NFC: Nanofibrillar cellulose
NF-H: Neurofilament heavy
NF-L: Neurofilament light
NF-M: Neurofilament medium
NOX: NADPH oxidase
NTA: Nanoparticle tracking analysis
PARP: Poly ADP (Adenosine Diphosphate)-Ribose Polymerase
PBS: Phosphate-buffered saline
PDGF: Platelet-derived growth factor
PTMs: Post-translational modifications
qPCR: Quantitative polymerase chain reaction
RNA: Ribonucleic acid
ROS: Reactive Oxygen Species
TEM: Transmission electron microscopy
TGF: Transforming growth factor
TNF: Tumor necrosis factor
TSG101: Tumor susceptibility gene 101
ULFs: Unit-length filaments
VEGF: Vascular Endothelial Growth Factor
Vim-/-: Vimentin knockout
Vim-/-Exo: Exosomes from vimentin knockout adipocyte progenitor cells
Vim-/-H-Exo: Exosomes from vimentin knockout hypo-osmotic stressed adipocyte progenitor cells
Vim-/-H+Exo: Exosomes from vimentin knockout hyper-osmotic stressed adipocyte progenitor cells
WB: Western blot
WCL: Whole cell lysate
WT: Wild-type
WT-Exo: Exosomes from wild-type adipocyte progenitor cells
WT-H-Exo: Exosomes from wild-type hypo-osmotic stressed adipocyte progenitor cells

WT-H+Exo: Exosomes from wild-type hyper-osmotic stressed adipocyte progenitor

1. Introduction

Chronic wounds have become a significant source of major mortality and morbidity, which lead to high medical costs and poor quality of life. Despite great progress in wound healing therapy, the traditional treatments mostly are ineffective and challenging. Exosomes with a size of 30-150 nm in diameter have the potential to promote tissue repair, due to their intrinsic features such as high stability, non-immune rejection, homing effect, easy control of dosage, and concentration (Hettich et al., 2020)(Cabral et al., 2018) (Yates et al., 2022). Exosomes as a sustained delivery method of growth factors, proteins, and nucleic acids are a promising strategy to promote wound healing and tissue regeneration (Ferreira & Gomes, 2019).

Vimentin is a cytoskeleton protein that plays an important role in biological functions at the cellular and molecular levels. Vimentin is particularly important during the wound healing process and our laboratory has shown that mice lacking vimentin (Vim-/-) have severely impaired wound healing (Battaglia et al., 2018). Interestingly, vimentin has been reported detectable in exosomes from different cell types which are called exosomal vimentin (Chen et al., 2016) (Sharma et al., 2018)(Adolf et al., 2019).

During the first part of this project, we revealed the active and necessary role of exosomal vimentin from adipocyte progenitors in promoting fibroblast proliferation, migration, and ECM secretion. Our results from *in vivo* and *in vitro* experiments present strong evidence that exosomal vimentin has a critical role in shortening the healing time and reducing scar formation. These findings suggest a novel role for exosomes in mediating wound repair by transferring cytoskeletal proteins to the wound site.

The composition, biogenesis, and secretion of exosomes are strongly influenced by environmental and cellular stress conditions (Liu & Su, 2019). In such an environment, exosomes can act as stress modifiers through changing gene expression and phenotypic behaviors of recipient cells. However, stress-induced changes in the composition of exosomal cargo are an efficient adaptive mechanism that helps cells to modulate intracellular stress conditions and send signals to influence the response of distant cells (Villarroya-beltri et al., 2014). It has been shown that vimentin as a hyperelastic network could disperse the local mechanical stress to a larger region in the cytoplasm to protect cells against mechanical damage (Mendez et al., 2014).

For the second part of the project, prompted by our previous findings, we investigated the role of exosomal vimentin in protecting cells against osmotic mechanical stress during wound healing. Our results showed that osmotic stress

increases the size and enhances the release of exosomes. More importantly, we realized that exosomal vimentin enhances wound healing by protecting fibroblasts against stress and inhibiting stress-induced apoptosis. This data could reveal exosomes either as a stress modifier to restore the osmotic balance or as a conveyer of stress to induce osmotic stress-driven conditions.

According to previous studies, while mass production of exosomes is challenging, three-dimensional spheroid culture stimulates the secretion of *in vivo*-like extracellular vesicles (Thippabhotla et al., 2019a) and specifically increases exosome secretion from mesenchymal stem cells (MSCs) (Kim et al., 2018)(Cha et al., 2018). Previously, it was reported that the production of EVs using bioreactors such as hollow-fiber bioreactors (Watson et al., 2016) and CELLine Adhere 1000 (CLAD1000) flask (Palviainen et al., 2019) (Mitchell et al., 2008) maximizes EV yield in comparison to conventional 2D cell cultures (Patel et al., 2019). In the third part of this study, we aimed to develop a three-dimensional (3D) experimental cell culture model using nanofibrillar cellulose (NFC) natural hydrogel in combination with CELLine Adhere 1000 (CLAD1000) flask to enhance exosomes production in an *in vitro* model. Our results showed that this method could significantly enhance exosome release while also increasing the delivery of exosomes to recipient cells.

2. Review of the Literature

2.1. Wound healing

Despite great advances in tissue engineering and regenerative medicine, still impaired wound healing is a challenging clinical problem that imposes a significant economic and quality of life burden on both patients and society. It is estimated that at least 1% of people from high economy countries face a complex wound in their life. In the United States, almost 2% of the total population is affected by chronic wounds (Sen, 2021).

Wound healing is a complex and dynamic biological process involving various cellular and molecular mechanisms. This process requires the synchronization of various cell types including systemic and local cells in the wound bed in sequential steps (Wilkinson & Hardman, 2020). The lack of a suitable animal model to precisely compare with the human condition is one of the difficulties in studying wound healing which hurdle translating data from experimental models to clinical trials. Currently, scientists try to gain better knowledge of mechanisms involved in wound healing by studying specific pathological pathways in animal models as well as analyzing data from human wound samples (Eming et al., 2014).

Skin is the largest organ in the body that has three main layers: epidermis, dermis, and hypodermis. The epidermis is the outer layer that withstands the harsh external environment. Keratinocytes are the main cells in this layer that produce keratin. The dermis is located under the epidermis and provides strength, immunity, and optimal nourishment to the epidermis. The dermis is rich in extracellular matrix (ECM) such as collagen fibers, blood vessels, mechanoreceptors, and different types of cells such as fibroblasts. Hypodermis underlies the dermis and is made up of a large adipose tissue reservoir that acts as an energy source to protect internal structures (Fig. 1) (Takeo et al., 2015; Rodrigues et al., 2019).

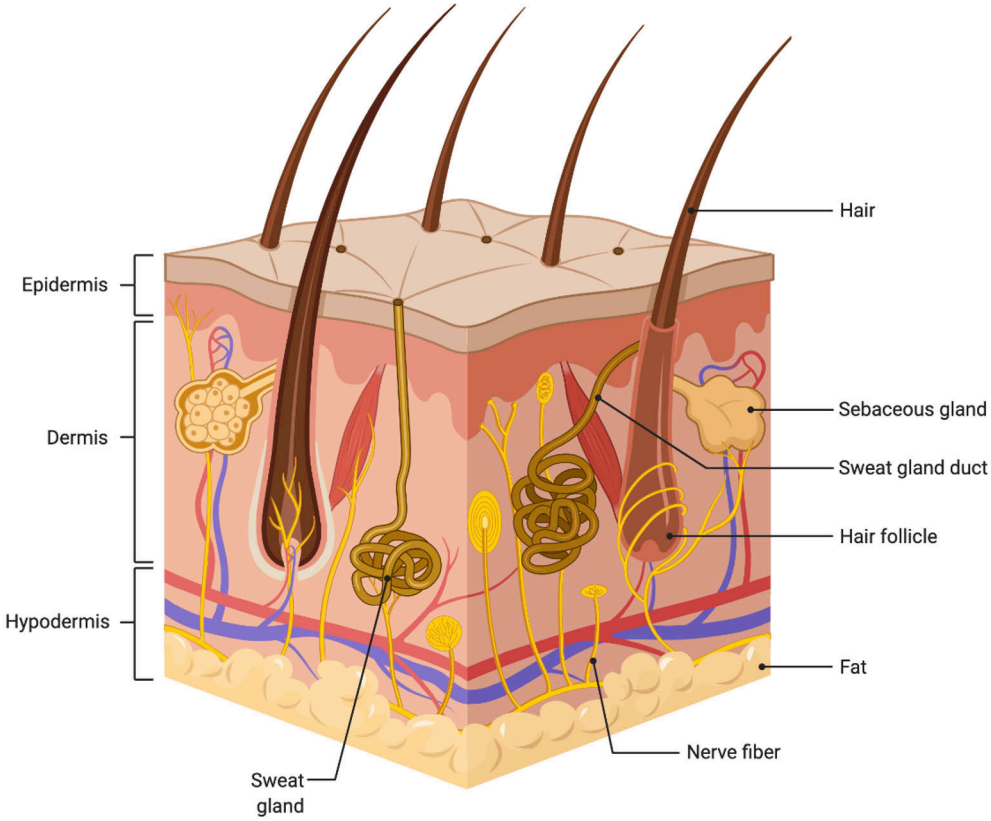

Figure 1. Schematic illustration of skin layers. The epidermis is the outer layer of skin that protects the outside environment. The dermis is highly vascularized which supports the skin's elasticity and strength. Hypodermis contains fat cells, blood vessels, and nerves. The image was created with BioRender.com.

The successful healing process is achieved through four main sequential but overlapping phases including hemostasis, inflammation, proliferation, and remodeling (Fig. 2) (Jeong, 2010).

The haemostasis phase is the first stage of wound healing that begins immediately after injury (Takeo et al., 2015). The haemostasis phase includes several stages including constriction of injured blood vessels to restrict the blood flow, platelet adhesion, degranulation, platelet aggregation, and fibrin clot formation. Platelets and ECM proteins are principal contributors to haemostasis. Platelets are activated to stop bleeding when interacting with ECM proteins such as fibronectin and collagen. Platelets recruit immune cells to the wound site and enhance cytokines and growth factor release (Wilkinson & Hardman, 2020). Pro-inflammatory cytokines and growth factors such as transforming growth factor

(TGF)-β, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF) are released by the clot and surrounding wound tissues (Ferreira & Gomes, 2019). Platelets are necessary for a successful tissue reconstitution. Platelet-rich plasma has been used in clinical applications to enhance the healing process (Eming et al., 2014).

The inflammatory phase begins in parallel with haemostasis and mobilizes local systemic defense response to the injury. The inflammatory phase is characterized by an increase in immune cell populations including sequential infiltration of neutrophils, macrophages, and lymphocytes to clear cell debris and microbes (Rodrigues et al., 2019). Continuous release of pro-inflammatory cytokines and growth factors by neutrophils, monocytes, and macrophages attracts leukocytes to the wound site and stimulates vasodilation (Wilkinson & Hardman, 2020b). In chronic wounds, the inflammation phase is prolonged and the healing process fails to progress. The prolonged inflammatory phase increases the infiltration of pro-inflammatory cells such as neutrophils and macrophages and deregulation of key proinflammatory cytokines, such as interleukin (IL)-1b and tumor necrosis factor (TNF)-α (Eming et al., 2014).

The proliferation phase overlaps with the inflammatory phase and involves re-epithelialization, angiogenesis, collagen synthesis, and ECM formation. During re-epithelialization, the proliferation of unipotent epidermal stem cells from the basement membrane and de-differentiation of epidermal cells repair the epidermis. The proliferation phase is characterized by the great activation of different cells such as keratinocytes, fibroblasts, macrophages, and epithelial cells to close the wound (Wilkinson & Hardman, 2020). Activated cells including keratinocytes produce matrix metalloproteinases (MMPs) to help other cells migrate to cover the exposed connective tissue and reconstitute the basement membrane. MMPs are multifunctional proteases that contribute to membrane shedding, ECM degradation, and chemokine production and release (Löffek et al., 2011). Keratinocytes as the major cellular component of the epidermis, undergo a partial epithelial-mesenchymal transition (EMT) to acquire a more invasive and migratory phenotype (Wilkinson & Hardman, 2020). Angiogenesis is triggered by hypoxia and occurs when endothelial cells proliferate and migrate to form new blood vessels. Macrophages support the migration of endothelial cells by producing MMPs to degrade the fibrin network and chemotactic factors such as TNF-α, and TGF-β (Wilkinson & Hardman, 2020). Simultaneously, fibroblasts proliferate and produce major components of ECM including collagen, glycosaminoglycans, and proteoglycans (Rybinski et al., 2014; Rodrigues et al., 2019).

The remodeling phase is the last stage of wound healing and is characterized by collagen remodeling, vascular maturation, and regression to restore normal tissue architecture which can last for several years (Guo & DiPietro, 2010; Rodrigues et al., 2019). This phase of wound healing determines whether scarring will occur or the wound will recur. As the remodeling of the wound progresses, reorganization of the ECM happens. During this process collagen III is lysed and partially replaces by stronger collagen I fibers (Rodrigues et al., 2019). Successful remodeling requires a perfect balance between collagen synthesis and degradation which is regulated by MMPs (Wilkinson & Hardman, 2020).

The wound healing process is ended when involved cells such as fibroblasts, macrophages, and endothelial cells experience apoptosis and leave the wound site while forming a scar (Wilkinson & Hardman, 2020).

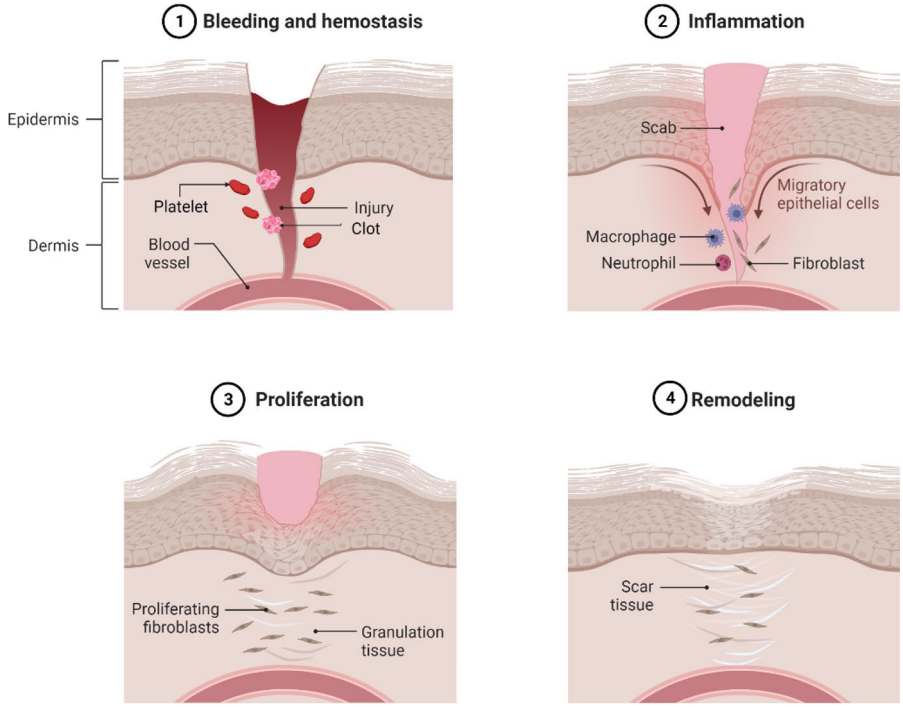

Figure 2. Schematic illustration of a normal wound healing process. Normal wound healing generally takes 4 to 6 weeks and includes four main sequential but overlapping steps: haemostasis, inflammation, proliferation, and remodeling. The image was adapted from (Tartaglia et al., 2021) and created with BioRender.com.

The outcome of normal wound healing is the restoration of a functional epidermal barrier and tissue repair which normally takes about 7- 14 days. However, under clinical conditions such as diabetes, vascular disease, radiation injury, altered immune system, or aging, interruption in one or several healing phases could link to impaired wound healing (Rybinski et al., 2014; Rodrigues et al., 2019).

Fibroblasts are spindle-shaped cells with a mesenchymal origin that are responsible for tissue haemostasis and wound healing. Under normal conditions, fibroblasts appear in the wound site at the end of the inflammatory phase and the beginning of the proliferative phase. Fibroblasts migrate to wound areas, proliferate, degrade fibrin clots, promote ECM production and mediate wound contraction (Bainbridge, 2013). When tissue is injured, surrounding fibroblasts differentiate into highly contractile cells called myofibroblasts. Myofibroblasts secret a high amount of ECM and reduce the size of the wound. On the other hand, excessive activity of myofibroblasts generates mechanical stress that leads to impaired wound healing and fibrosis (Darby & Hewitson, 2007).

Tumors have been considered as non-healing wounds. The wound healing process shares similar characteristics with chronic fibrosis and tumor progression such as the mutual presence of the EMT process and myofibroblastic differentiation processes (Rybinski et al., 2014). EMT occurs during physiological or pathological processes such as embryogenesis, wound healing, and cancer development and is characterized by the transition of the cells from sedentary to the migratory state (Fig. 3). This process is mediated by inflammatory cells and fibroblasts which support the mesenchymal architecture during the re-epitalization phase in wound healing (Barriere et al., 2015). Myofibroblast differentiation and the EMT process determine the fate of the wound either towards the healing or fibrosis and tumor progression (Fig. 4) (Darby & Hewitson, 2007).

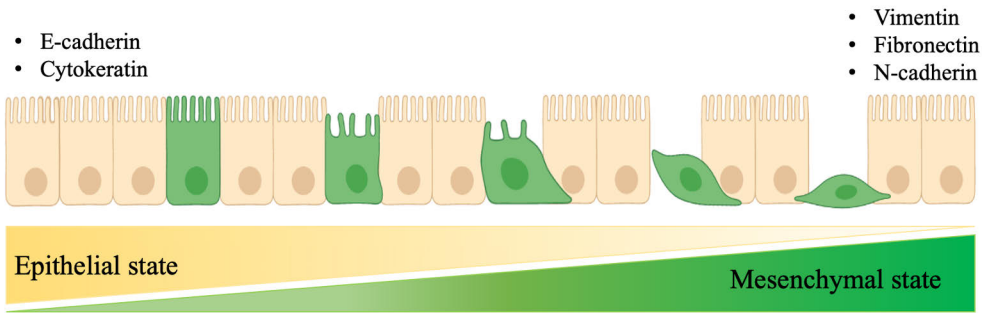

Figure 3. Epithelial-mesenchymal transition. During EMT, epithelial cells lose their cell membrane epithelial markers such as E-cadherin and cytokeratin and gain mesenchymal markers such as vimentin, fibronectin, and N-cadherin. The image was adapted from (Barriere et al., 2015) and created with BioRender.com.

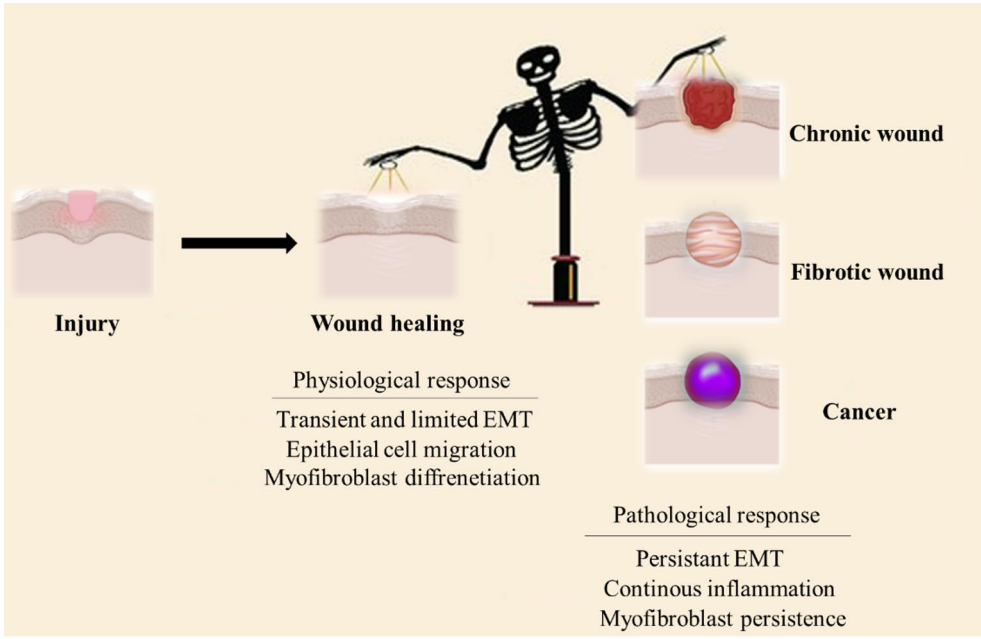

Figure 4. The fate of the injury in physiologic and pathological conditions. The outcome of a desirable wound healing process is tissue regeneration that restores normal tissue function. A prolonged, incomplete, and uncoordinated healing process result in impaired wound healing. An impaired or excessive wound healing process causes chronic inflammation and fibrosis which contribute to tumor formation and cancer. EMT: Epithelial-mesenchymal transition, ECM: extracellular matrix. The image was adapted from (Rybinski et al., 2014) and created with BioRender.com.

The impaired healing process can lead to either an excess of scar formation (hypertrophic scar or keloid) or ulcerative skin (chronic wound) (Eming et al.,

2014). Cutaneous wounds are classified into two categories; acute wounds which are caused by environmental factors and heal normally, and chronic wounds which are usually caused by metabolic disorders and do not heal normally (Fig. 5) (Irfan-maqsood, 2018).

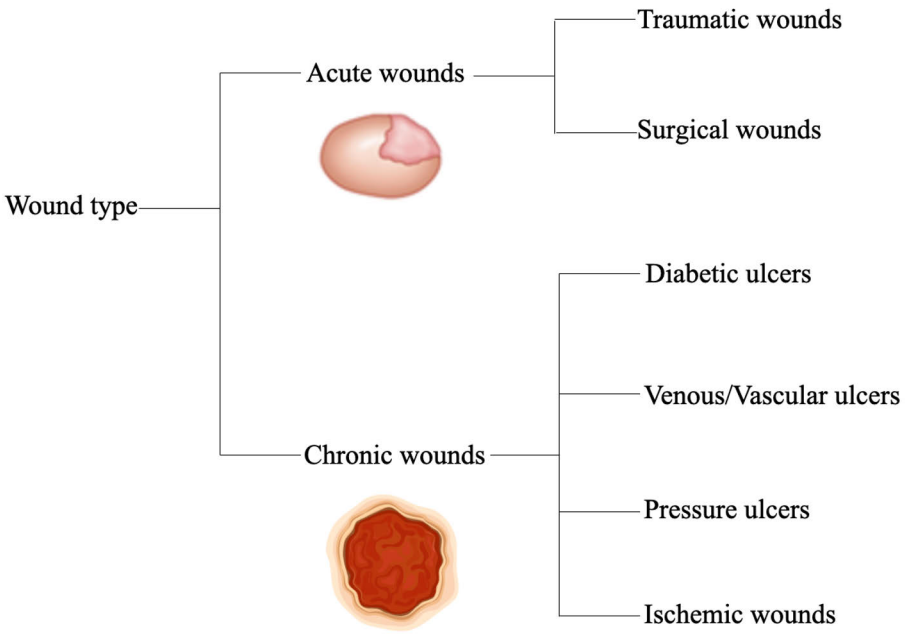

Figure 5. Wound types include acute and chronic wounds. Cutaneous wounds are classified into acute and chronic wounds. Acute wounds usually are caused by environmental damage and heal normally. Chronic wounds can be the results of metabolic disorders. The image was adapted from (Irfan-maqsood, 2018) and created with BioRender.com.

2.1.1. Current treatments for wound healing

Wound healing therapies are categorized into traditional and modern therapies. Traditional therapies include herbal and animal-derived compounds, living organisms, silver, and traditional wound dressings. Traditional therapies are cost-effective and affordable approaches and mostly try to minimize the spread of microorganisms and accelerate healing time. Traditional wound dressing including products such as gauzes, cotton wool, and natural or synthetic bandages have been used frequently in wound care applications (Pereira & Ba, 2016; Oliveira et al., 2020). However, there are limitations associated with traditional wound dressing such as failure to provide a moist environment and

the possibility of dryness and adhesion to the wound bed which damage the epidermis and causes trauma. To tackle these challenges, these products are commonly used as a secondary or in combination with other treatments (Pereira & Ba, 2016; Monika et al., 2022).

With the continuous advancement in the field of regenerative medicine and biomaterial sciences, modern wound healing therapies have been developed to treat wounds more efficiently. The most common modern wound healing therapies are described in table 1.

Table 1. The most common wound healing therapies. PDGF: platelet-derived growth factor, FGF: fibroblast growth factor, EGF: epidermal growth factor.

Method	Example	Reference
Advanced wound dressings	Nanomaterial-based dressing Drug-containing dressing Skin substitutes	(Boateng & Catanzano, 2015)
Wound physical therapies	Oxygen wound therapy Negative pressure wound therapy Shock wave wound therapy Photobiomodulation	(Oliveira et al., 2020)
Exogenous growth factor-based therapy	PDGF, FGF, and EGF	(Oliveira et al., 2020)
Cell-based therapy	Embryonic stem cells Induced pluripotent stem cells Mesenchymal stem cells (MSCs) Adipose-derived stem cells (ASCs) Hematopoietic stem cells	(Kanji & Das, 2017)

2.1.2. Stem cell therapeutics in wound healing

Stem cells are undifferentiated cells that have the ability to self-renewal as well as differentiation into any cell type. Stem cells are classified into embryonic stem cells and adult stem cells. While adult stem cells can be isolated from almost all tissues, embryonic stem cells are isolated only from the inner cell mass of blastocytes. In the skin, epidermal stem cells are located in the bulge of the hair follicle, the base of the sebaceous gland, and the basal layer of the epidermis. Local adipocyte progenitor cells and melanocyte progenitors contribute also to wound repair (Eming et al., 2014). Previous studies showed that different phases of cutaneous wound healing are mediated by adult stem cells proliferation and signaling while non-healing wound conditions such as diabetes can affect the

functionality of stem cells (Coalson et al., 2019). Especially adipose-derived stem cells (ASCs) have attractive characteristics that make them suitable candidates for tissue regeneration such as abundant supply, ease of isolation, high yield, and extensive proliferative capacities. ASCs promote skin repair by two mechanisms, firstly by their ability to migrate and differentiate into skin cells to repopulate injured skin secondly by activating human dermal fibroblast proliferation by secretion of growth factors and signaling activation in the re-epithelialization phase of wound healing (Fig. 6) (Hassan et al., 2014).

Various wound healing factors have been detected in the secretion profile of ASCs such as TGF-β, PDGF, FGF2, insulin-like growth factor (IGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), keratinocyte growth factor (KGF), fibronectin, and collagen (Hassan et al., 2014). It has been shown that these factors enhance the formation of new vasculature in the wound bed and stimulate the recruitment, migration, and proliferation of fibroblasts and keratinocytes. Therefore, ASCs accelerate angiogenesis, epithelialization, and wound remodeling through paracrine secretion during the wound repair process (Hassan et al., 2014).

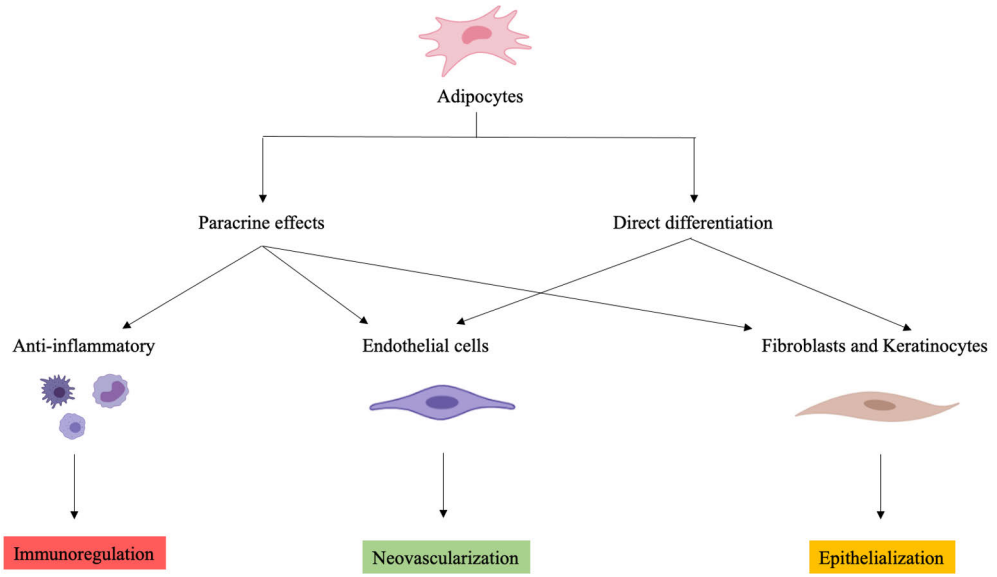

Figure 6. Mechanism of skin repair by adipose-derived stem cells (ASCs). ASCs accelerate wound healing through migrating and differentiating into the skin cells or activating the skin cells by secretion of growth factors through paracrine secretion. The image was adapted from (Hassan et al., 2014) and created with BioRender.com.

2.2. Extracellular vesicles

There are two tightly connected vesicular transport systems in eukaryotic cells: intracellular vesicle transport and extracellular vesicle transport system. In the intracellular vesicular system, various endomembrane organelles such as Golgi apparatus, endoplasmic reticulum (ER), endosomes, and lysosomes, in connection with cytoskeletal proteins are responsible for cytoplasmic trafficking of biomolecules. The extracellular vesicle transport system mediates by the secretion of extracellular vesicles (Salimi et al., 2020; Yates et al., 2022).

Extracellular vesicles (EVs) are lipid bilayer membrane-enclosed particles that are secreted by cells into the extracellular space (Salimi et al., 2020; Yates et al., 2022). Previously, EVs have been considered just as garbage bags to dispose cellular waste. However, recent studies showed that EVs act as a highly regulated mode of communication between and among cells and tissues (Cabral et al., 2018). EVs interact and transport their cargo to the recipient cells and affect the status of the cells. The cargo of EVs is cell type-dependent and is modulated by the physiological or pathological state of the parental cells and their surrounding environmental stimuli (van Niel et al., 2018). It has been shown that the packaging of EVs cargo is selective and consists of different biomolecules such as proteins, lipids, DNA, mRNA, and miRNA (van Niel et al., 2018). For example, ubiquitination is one of the mechanisms by which proteins are selectively targeted into the EV pathways (Stahl & Raposo, 2019). Also, it has been reported that some miRNAs contain a targeting sequence that may regulate the selective packaging of RNAs by selectively recruiting specific miRNAs into newly forming exosomes (Stahl & Raposo, 2019). EVs are enriched in different kinds of lipids including phosphatidylserine (PS), ganglioside, cholesterol, glycosphingolipids, and ceramide (Yates et al., 2022). EVs can be isolated from all body fluids such as urine and breast milk as well as dissociated tissues and cell culture supernatants (Doyle & Wang, 2019; Yates et al., 2022).

For successful communication, EVs transport their content to the local or distant recipient cells through different mechanisms including direct membrane fusion which is followed by the activation of surface receptors and signaling pathways, clathrin-mediated or clathrin-independent endocytosis such as macropinocytosis and phagocytosis or endocytosis by caveolae and lipid rafts (Fig. 7) (van Niel et al., 2018; Ratajczak & Ratajczak, 2020).

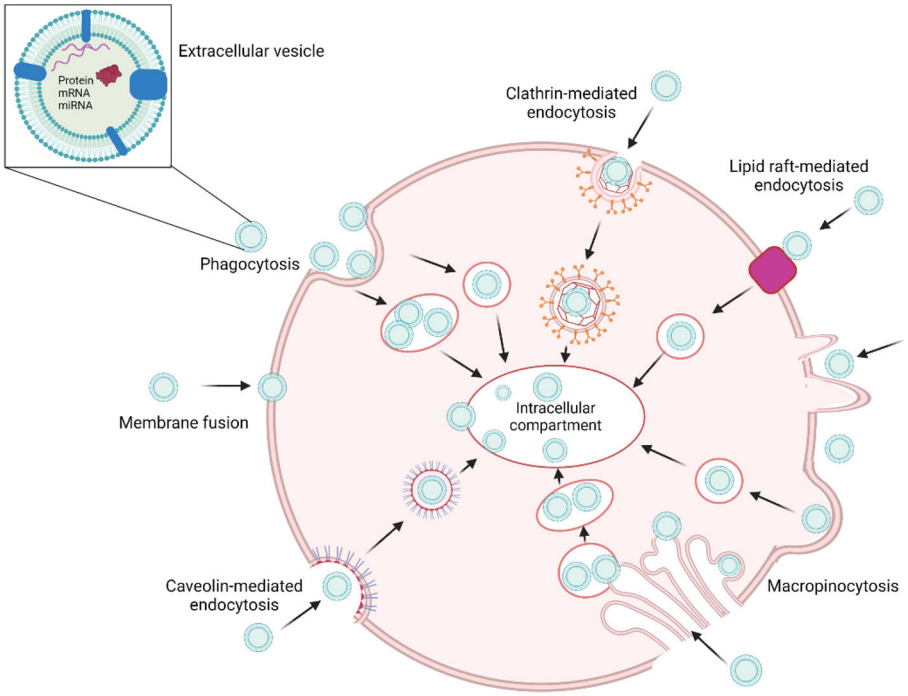

Figure 7. Schematic illustration of extracellular vesicle uptake mechanisms. EVs interact with their recipient cells through different mechanisms such as membrane fusion, clathrin-mediated endocytosis, macropinocytosis, and phagocytosis. The image was adapted from (Ann Mulcahy et al., 2014) and created with BioRender.com.

The field of the extracellular vesicle is a fast-growing and fairly new field of study and our knowledge from the field is an ongoing process. There have been continuous changes in EVs nomenclature and classification, collection, separation, characterization, and functional assays. In 2014 the International Society for Extracellular Vesicles (ISEV) proposed a guideline for the Minimal Information for Studies Extracellular Vesicles ("MISEV") (Lötvall et al., 2014). Although most of the MISEV2014 recommendations are still valid, with the discoveries and developments in the field, the guideline was updated in 2018 to provide more detailed protocols to study EVs (Théry et al., 2018).

EV is a generic umbrella term for all the naturally released lipid bilayer particles from the cells which cannot replicate and according to MISEV 2018, the term EV should be used when the subpopulation of the EV type cannot be ascertained (Théry et al., 2018). However, according to MISEV 2018, with emerging other subtypes of extracellular vesicles and difficulties to assign EVs to a particular biogenesis pathway, EVs can be further categorized according to

their physical characteristics such as size or density, biochemical composition, and/or descriptions of conditions or cell of origin (Kowal et al., 2016; Théry et al., 2018).

In the past, EVs were classified according to their route of formation and size into three subtypes: exosomes (30–150 nm), microvesicles (100–1000 nm), and apoptotic bodies (1000–5000 nm). Despite the unique biogenesis pathway for each subtype, there is a substantial overlap in the composition, density, and size of different subtypes which makes the study of the individual EV subsets highly challenging (Doyle & Wang, 2019). Recently, new subtypes of EVs have been introduced such as exomers, oncosomes, and migrasomes (Fig. 8) (Gurunathan et al., 2021).

Microvesicles also known as ectosomes and microparticles formed by direct outward budding of the plasma membrane. Microvesicles contain cytosolic and plasma membrane-associated proteins including tetraspanins, cytoskeletal proteins, heat shock proteins, integrin, active proteases, and multiple small GTPases (Clancy et al., 2021; Ratajczak & Ratajczak, 2020).

Apoptotic bodies are formed from the outwards blebbing of the plasma membrane when cells undergo apoptosis. Apoptotic bodies contain organelles and organelles fragments such as mitochondria, nucleus, Golgi apparatus, endoplasmic reticulum, chromatin, and some glycosylated proteins. In direct contrast with exosomes and microvesicles, the content of apoptotic bodies is quite similar to the cell lysate (Cabral et al., 2018).

Exomers are protein complex with an average size of 35 nm. Exomers mediate the sorting of specific plasma membrane proteins into vesicles at the trans-Golgi network. Exomers facilitate the vesicle transport from the trans-Golgi network to the plasma membrane and its absence leads to the retention of a set of selected cargoes in trans-Golgi (Ramirez-Macias et al., 2018; Anand et al., 2021; Moro et al., 2021).

Oncosomes are 100–400 nm membrane-derived extracellular vesicles that are secreted by cancer cells. However, in other cases, large oncosomes with a size of 1–10 μm can be formed. Oncosomes contain specific cargo relating to tumor formation and transferring oncogenic messages which can control tumor progression. Oncosomes are formed as byproducts of non-apoptotic cells by outward shedding of the cellular membrane (Ciardiello et al., 2020; Gurunathan et al., 2021).

Migrasomes are 500-3000 nm pomegranate-like structures that produce by migrating cells. They play important roles as carriers of damaged mitochondria, releasing signaling molecules and lateral transfer of mRNA or proteins (Yu & Yu, 2021; di Daniele et al., 2022).

According to MISEV 2018 (Théry et al., 2018), it is preferred to use the name "extracellular vesicles" over exosomes since it is challenging to ensure that a specific subtype of EVs such as exosomes is present in a sample without contamination with other EVs subtype. Since the guideline is achievable as of this writing, to be compliant with the MISEV 2018 guideline, the used term "exosome" in this thesis refers to the general term small extracellular vesicles (50-150 nm) isolated by the commonly accepted methods such as ultracentrifugation and ultrafiltration expressing specific markers such as tetraspanins.

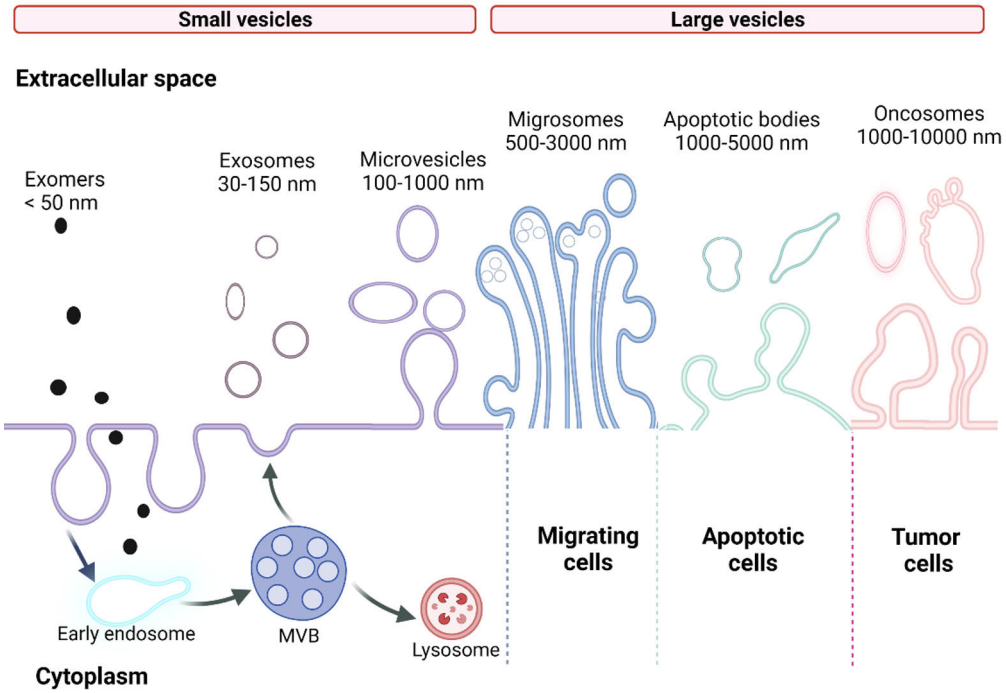

Figure 8. Schematic illustration of different subtypes of EVs. Exomeres are non-membranous nanoparticles smaller than 50 nm. Exosomes are the smallest EV subtype that generate from the infusion of multivesicular bodies (MVBs) with the plasma membrane. Microvesicles are mid-sized EVs that form by the outward budding of the plasma membrane. Migrasomes are pomegranate-like structures that are released from the tip of retraction fibers of migrating cells. Apoptotic bodies are irregularly shaped structures formed from the outwards budding of plasma membrane when cells undergo apoptosis. Oncosomes are large-sized EV subpopulations that generate by large protrusions of the plasma membrane of cancer cells. The image was adapted from (di Daniele et al., 2022) and created with BioRender.com.

2.2.1. Exosomes

EVs were first reported in chondrocytes (Anderson, 1967; Bonucci, 1969), platelets (Wolf, 1967) and gram-negative bacteria in the 1960s (Knox et al., 1966; Work et al., 1966). In 1983, vesicles containing peptides were observed in sheep immature red blood cells (Pan & Johnstone, 1983), which were later in 1987 called 'exosomes' (Johnstone et al., 1987). In 1996, Stoorvogel et al. discovered that exosomes may have a role in immune regulation and regulating extracellular microenvironment (Raposo et al., 1996). Later in 2007, scientists found that exosomes can mediate cell-cell communications by transferring and exchanging genetic information (van Niel et al., 2006; Valadi et al., 2007). Further investigations demonstrated that exosomes by carrying specific cargo are involved in cellular processes such as immune responses, cell migration and differentiation, tumor invasion, and autophagy (Baixauli et al., 2014; Kalluri & LeBleu, 2020; Xing et al., 2021).

Exosomes are the smallest subpopulation of EVs that are formed via the endosomal route. They were originally considered a means of eliminating unwanted materials from the cells (Johnstone et al., 1987). However, it has been shown that EVs are the mediators of intracellular communication in physiological and pathological conditions such as pregnancy, cardiovascular diseases, cancer, immune responses, and tissue repair (Kalluri & LeBleu, 2020). The process of exosome biogenesis starts with the formation of early endosomes through inward budding of the plasma membrane and then their maturation to multivesicular bodies (MVBs). MVBs can either fuse with lysosomes for degradation or fuse with the plasma membrane to form and release exosomes into the extracellular space (Wauben, 2015). The mechanism behind MVB formation, cargo sorting, and finally exosome release is regulated by multiple pathways including endosomal sorting complexes required for transport (ESCRT)-dependent pathway and ESCRT independent pathway. Some proteins such as tumor susceptibility gene 101 (TSG101), programmed cell death 6 interacting protein (Alix), and heat shock protein 70 (HSP70) are parts of the ESCRT pathway which are detected in exosomes regardless of the originated cell source. In ESCRT independent pathway, exosome release depends on the sphingomyelinase enzyme (Fig. 9). Even though the content of exosomes is highly dependent on the isolation method, tetraspanins including CD9, CD63, and CD81 are considered to be enriched in exosomes when isolation is based on size difference (Doyle & Wang, 2019).

In addition to the proteins and nucleic acids, lipids are a critical component of all EVs. Lipids play important role in EVs structure, regulatory functions,

biogenesis, release, cellular targeting, and uptake (Donoso-Quezada et al., 2021; Yates et al., 2022). There is another pathway of EV biogenesis different from the ESCR-dependant and independent mechanisms. In this pathway, the composition of endosomal membrane lipid changes to form subdomains called lipid rafts. It has been shown that lipid rafts and ceramides induce exosome formation and release of exosome cargo into the extracellular space (Elsherbini & Bieberich, 2018; Skryabin et al., 2020).

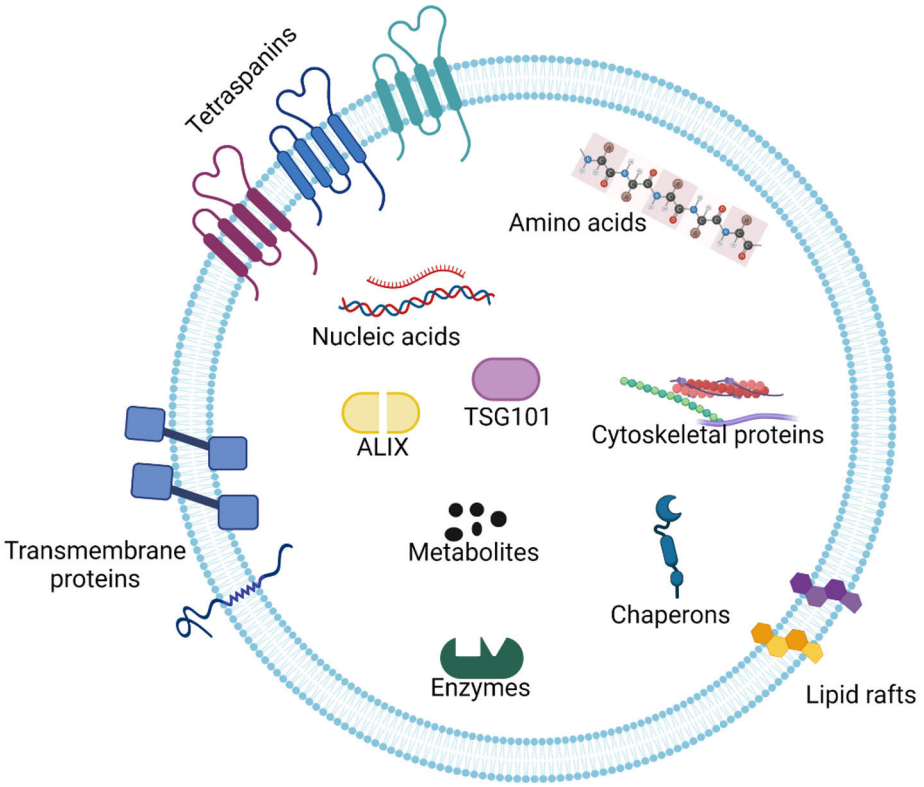

Figure 9. General representation of the exosome structure and content. Exosomes have a typical lipid bilayer membrane and carry nucleic acids including RNA and DNA, cell surface markers such as integrin, tetraspanins (CD63, CD9, and CD81), proteins including signaling proteins, enzymes, cytoskeletal proteins, and chaperones. The image was adapted from (Kalluri et al., 2020) and created with BioRender.com.

2.2.2. Exosomes and wound healing

EVs, contribute not only to intercellular communication but also to other processes such as cell proliferation, apoptosis, migration, invasion, and differentiation. Some applications may harness the intrinsic properties of EVs such as regeneration, immune modulation and tumor, and pathogen suppressions (Nederveen et al., 2021; Yates et al., 2022).

MSCs by having the ability to self-renewal and differentiate into different cell types are an attractive choice for clinical applications (Pittenger et al., 2019). Human MSCs promote cutaneous wound healing by affecting their neighbor cells through the endocrine signaling pathway (local signals between the similar cell type), paracrine signaling pathway (local signals between different cell types), and expression of different growth factors (Hu et al., 2019; Nikfarjam et al., 2020).

Increasing evidence suggests that MSC-derived exosomes (MSC-Exos) contribute to tissue regeneration and wound healing (Nikfarjam et al., 2020). The application of MSC-Exos has several advantages over MSCs including stability, safety, and easy formulation (Hu et al., 2019). MSCs-derived exosomes contribute to different phases of wound healing (Hettich et al., 2020). In haemostasis phase, in an *in vitro* model, MSC-EVs have been shown to induce blood coagulation (Silachev et al., 2019; Zeng & Liu, 2021). In the inflammation phase, MSC-Exos significantly regulate immunomodulatory response by affecting inflammatory cells, cytokines, and enzymes through their cargo (Lo Sicco et al., 2017). In the proliferation phase, MSC-Exos activate neoangiogenesis and promote re-epithelialization, proliferation, and migration of the skin cells (Shabbir et al., 2015). In the remodeling phase, MSC-Exos regulate ECM re-synthesis by regulating collagen I and III production in the early and late stages of wound healing (Zhang et al., 2016; Wang et al., 2017). Furthermore, it has been shown that local autocrine secretion of exosomes significantly enhances cell migration by delivering the ECM cargo and promoting cell adhesion (Sung et al., 2015).

As we discussed earlier, ASCs promote angiogenesis, epithelialization, and wound remodeling through paracrine secretion (Hassan et al., 2014). It has been shown that cutaneous wounds with fat layers heal faster than wounds without fat due to the presence of fat and higher distribution of exosomes (Hassan et al., 2014; Hu et al., 2016). Specifically, in the cutaneous wound, ASC-derived exosomes (ASC-Exos) are internalized by fibroblasts and modify their functions such as proliferation, migration, and ECM production towards faster healing (Hu

et al., 2016). These findings suggest that MSC-Exos and particularly ASC-Exos can be considered a promising novel therapeutic tool for soft tissue wound healing.

2.3. Cytoskeletal proteins

The cytoskeleton is an interconnected network consisting of filamentous polymers and regulatory proteins that play a key role in cell content arrangement, cell shape, movement, attachment to other cells or extracellular matrices, and transporting intracellular cargo (Fletcher & Dyche Mullins, 2010; Pegoraro et al., 2017; Pollard & Goldman, 2018; Mogessie et al., 2019; Mactaggart & Kashina, 2021; Chuang & Chen, 2022; Ndiaye et al., 2022). Cytoskeleton proteins consist of three major components: microfilaments (actin cytoskeleton), microtubules (tubulin cytoskeleton), and intermediate filaments (IF) (Fig. 10) (Fletcher & Dyche Mullins, 2010; Pegoraro et al., 2017; Pollard & Goldman, 2018; Mogessie et al., 2019; Mactaggart & Kashina, 2021; Chuang & Chen, 2022; Ndiaye et al., 2022).

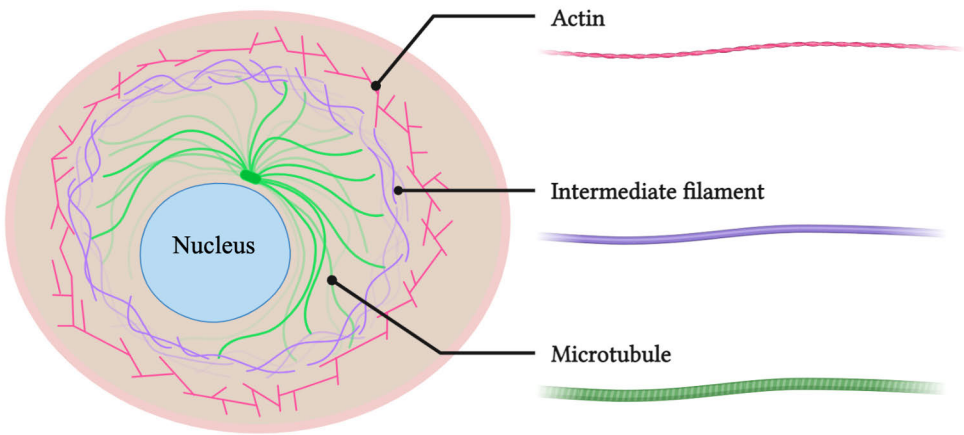

Figure 10. Schematic illustration of cytoskeletal proteins. The cytoskeleton maintains the location of internal cellular structures such as the nucleus and cell organelles. It provides structural stability and cell movement. The image was created with BioRender.com.

2.3.1. Intermediate filaments

Intermediate filaments are strong but highly flexible polymers that are distinguished by their size with a diameter between 10 to 12 nm from actin

microfilaments (6–8 nm) and microtubules (25 nm). IFs are composed of homologous proteins with a size between 40 to 240 kDa (Chang & Goldman, 2004; Depianto & Coulombe, 2004; Eriksson et al., 2009; Goldman, 2018; Dutour-Provenzano & Etienne-Manneville, 2021; Redmond & Coulombe, 2021). In contrast to microfilaments and microtubules, which are evolutionarily highly conserved, IFs are cell-type specific and share just 20% sequence identity. Self-assembly is the main characteristic feature of all IF proteins that is determined by a central α-helical domain comprised of 310 and 350 amino acids (Fuchs & Weber, 1994).

IFs play important role in regulating cell shape and mechanical integrity. IFs are encoded by more than 70 genes in humans and apart from lamins (type V) that can be found in the nucleus, the rest of IFs are located in the cytoplasm. IFs are classified according to their gene structure and nucleotide homology of their central alpha-helical domain (Herrmann & Aebi, 2016). According to this classification, there are five groups of IFs including type I (acidic keratin), type II (basic keratin), type III (vimentin, desmin, glial fibrillary acidic protein, peripherin, syncoilin), type IV (neurofilament and alpha-Internexin) and type V (Lamins) (Table 2) (Herrmann & Aebi, 2016; Goldman, 2018; Dutour-Provenzano & Etienne-Manneville, 2021; Redmond & Coulombe, 2021).

Generally, IFs are composed of a conserved central alpha-helical rod domain consisting of three sub-helices including coil 1A, 1B, and coil 2. Coil 1A and 1B are connected by linker L1 and coil 1B is connected to coil 2 by linker L2 (Fig. 11). The rod domain is flanked by a non-alpha-helical amino head and carboxyl-terminal domains. During filament formation, the parallel assembly of two rod domains forms a coiled-coil dimer. Then two dimers associate laterally in opposite directions to each other and form a tetramer. The lateral arrangement of eight tetramers forms unit-length filaments (ULFs) which then assemble in an end-to-end fusion format and form the filaments (Lowery et al., 2015; Pollard & Goldman, 2018). Contrary to actin and microtubules which normally are polarized, the antiparallel arrangement of IFs results in non-polar filaments (Lowery et al., 2015). IFs play important roles in protecting cells against cellular stress and regulating cell death, growth, proliferation, and migration (Chang & Goldman, 2004; Depianto & Coulombe, 2004; Eriksson et al., 2009; Goldman, 2018; Dutour-Provenzano & Etienne-Manneville, 2021; Redmond & Coulombe, 2021).

Table 2. Five main classes of intermediate filaments and their location. CNS: central nervous system; GFAP: glial fibrillary acidic protein; NF-H: neurofilament heavy; NF-L: neurofilament light; NF-M: neurofilament medium.

Class	Type	Distribution
I	Acidic keratin	Epithelial cells
II	Basic keratin	Epithelial cells
III	Vimentin	Mesenchymal cells
	Desmin	Muscle cells
	GFAP	Glial cells
	Peripherin	Peripheral neurons
	Syncoilin	Muscle cells
IV	NF-L	CNS neurons
	NF-M	
	NF-H	
	a-Internexin	
V	Lamins	Nucleus

2.3.2. Microfilaments (Actin Cytoskeleton)

The actin cytoskeleton is a dynamic network composed of actin polymers and actin-binding proteins. The actin cytoskeleton is responsible for cell structural organization, cell motility and contractility, vesicle and organelle movement, and phagocytosis (Fletcher & Dyche Mullins, 2010; Pegoraro et al., 2017; Pollard & Goldman, 2018; Mogessie et al., 2019; Mactaggart & Kashina, 2021; Chuang & Chen, 2022; Ndiaye et al., 2022). Besides its structural role, actin can move to the nucleus and regulates gene expression as a component of chromatin-remodeling complexes and core histone complexes. The dynamic structure of the actin cytoskeleton including assembly and disassembly of actin filaments is regulated by signaling pathways and actin-associated proteins (Fletcher & Dyche Mullins, 2010; Pegoraro et al., 2017; Pollard & Goldman, 2018; Mogessie et al., 2019; Mactaggart & Kashina, 2021; Chuang & Chen, 2022; Ndiaye et al., 2022).

2.3.3. Microtubules (tubulin cytoskeleton)

Microtubules are the stiffest cytoskeletal proteins that facilitate all important events leading to cell proliferation, cell migration and cell resist compression through their role in cell mechanics, intracellular trafficking, and signaling (Fletcher & Mullins, 2010). Moreover, microtubules make up the internal structure of fundamental units of motion in living cells such as cilia and flagella. Furthermore, microtubule dynamics can regulate different cellular processes such

as chromosome movement during cell division, intracellular macromolecular assemblies, and movement of vesicles and organelles (Pollard & Goldman, 2018).

2.3.4. Vimentin

Vimentin is a 54 kDa, 466 amino acid type III intermediate filament protein (UniProtKB-P08670) encoded by the vimentin gene (VIM) (Ostrowska-Podhorodecka et al., 2022; Ridge et al., 2022). Vimentin is an evolutionarily highly conservative protein among vertebrates and is generally expressed in cells of mesenchymal and ectodermal origin (Ridge et al., 2022; Paulin et al., 2022). Vimentin is initially broadly expressed in the embryonic development phase. In adult tissues, vimentin expression is restricted in certain cell types including fibroblasts, endothelial and hematopoietic cells (Paulin et al., 2022).

Vimentin cytoskeletal network consists of stress-resistance filaments. In the cells, these filaments extend in the cytoplasm from the nuclear periphery to the cell membrane (Pérez-Sala et al., 2015; Ridge et al., 2022). However, vimentin can also be found in the extracellular space in smaller and non-filamentous forms. Extracellular vimentin is localized at the surface of the plasma membrane or released in the extracellular environment under different physiological and pathological conditions (Shigyo et al., 2015; Shigyo & Tohda, 2016; Fasipe et al., 2018; Yu et al., 2018; Patteson et al., 2020; Huijbers et al., 2021; Suprewicz et al., 2021; Paulin et al., 2022; van Beijnum et al.,2022).

Similar to other IFs, vimentin monomers assemble into parallel dimers and then turn into antiparallel tetramers as the structural units for vimentin polymerization which form eight tetramers, ULFs, and finally 10 nm mature filament (Fig. 12) (Pérez-Sala et al., 2015). In physiological conditions, vimentin spontaneously assembles into 10 nm diameter filaments (Ostrowska-Podhorodecka et al., 2022).

The assembly/disassembly of vimentin regulates its biochemical and structural properties which is a requirement for vimentin cellular functions such as cell migration and cell division (Paulin et al., 2022). Vimentin expression is controlled both at the promoter level through interacting with a set of activating and inhibiting elements and epigenetic level through DNA and histone methylation, chromatin modifications, microRNAs, and long noncoding RNAs. In cell culture models, vimentin expression is regulated by the cell cycle, growth factors such as TGFβ1, PDGF and FGF, and INF-γ cytokine (Paulin et al., 2022). Moreover, the function, structure, and localization of the vimentin network are regulated by post-translational modifications (PTMs) such as citrullination, phosphorylation, ADP ribosylation, SUMOylation, and O-GlcNAcylation (Pérez-

Sala et al., 2015) (Kraxner et al., 2021; Paulin et al., 2022). Specifically, vimentin can exist in both non-phosphorylated and phosphorylated forms while phosphorylation at specific sites by various kinases causes filament disassembly and depolymerization. Phosphorylation regulates the dynamic reorganization of the vimentin network under different conditions such as cell spreading, cell division, and motility (Inagaki et al., 1989; Robert et al., 2015).

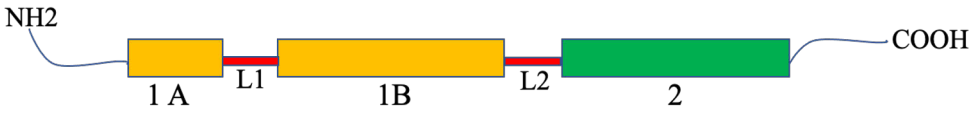

Figure 11. Illustration of the domain organization of vimentin. The central alpha-helical rod domain consists of three sub-helices including coil 1A, 1B, and coil 2. Coil 1A and 1B are connected by linker L1 and coil 1B is connected to coil 2 by linker L2. Each monomer has an amino-terminal head and a carboxyl-terminal tail.

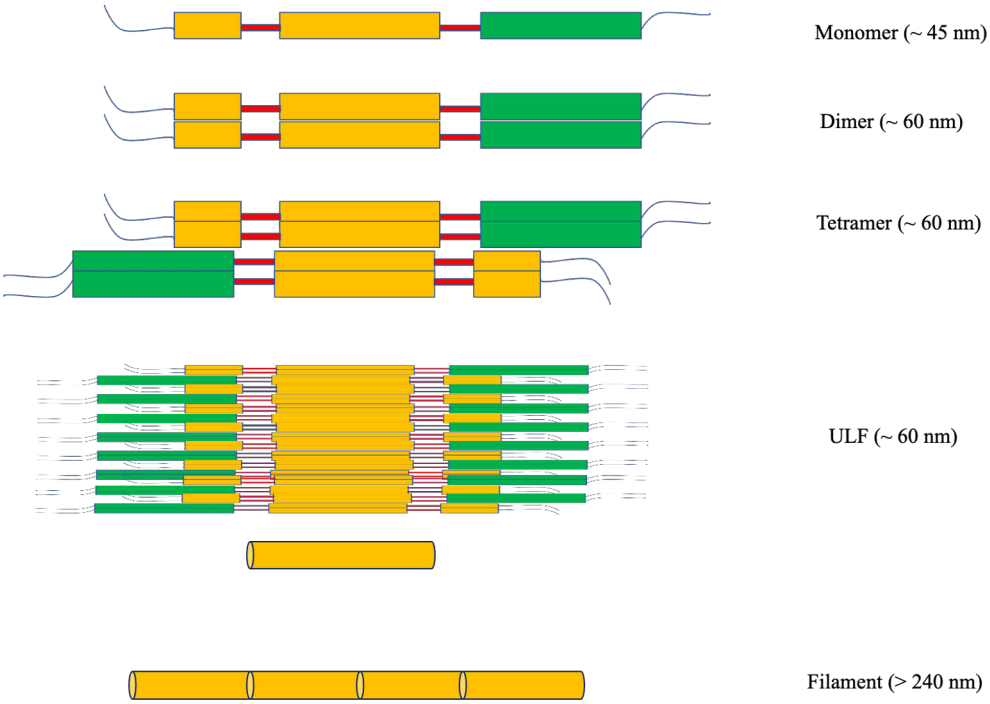

Figure 12. Schematic illustration of the molecular structure of vimentin (not to scale). Parallel alignment of two IF monomers form a coiled-coil dimer. Antiparallel alignment of two dimers forms tetramer. Unit length filaments (ULFs) are formed by the lateral arrangement of eight tetramers which then form a filament by the end-to-end elongation. The image was adapted from (Hohmann & Dehghani, 2019).

According to Human Protein Atlas database (https://www.proteinatlas.org/ENSG00000026025-VIM/tissue), vimentin is expressed in the majority of the tissues such as lung, brain, skin, bone marrow, and lymph nodes (Fig. 13) (Denielsson et al., 2018).

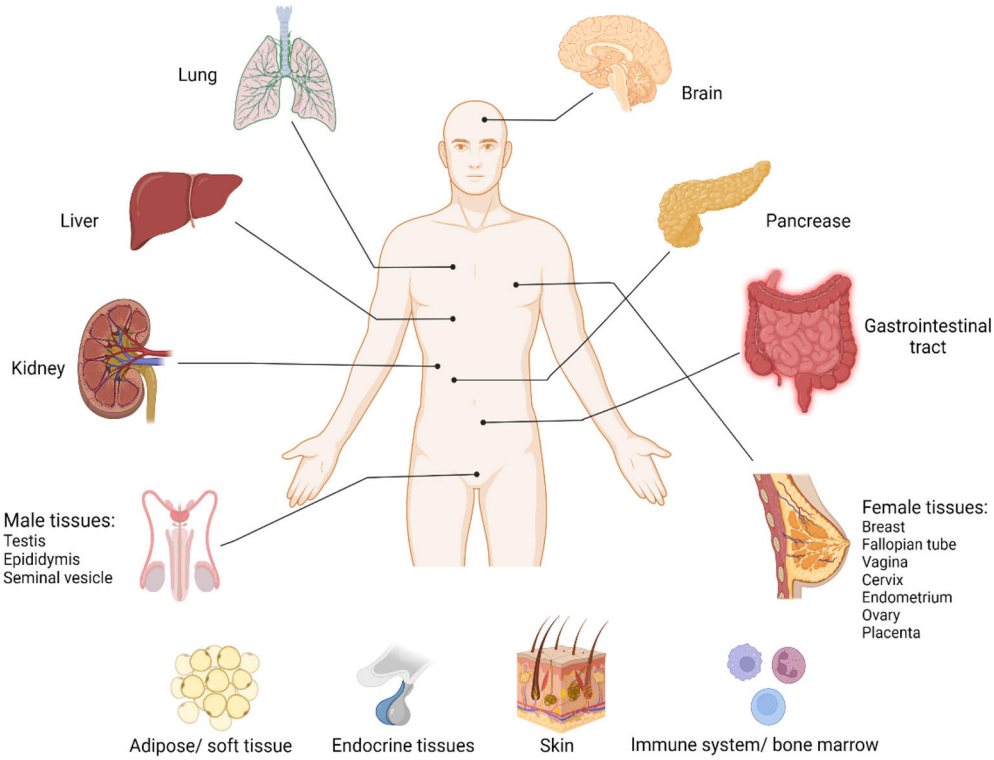

Figure 13. Examples of tissues identified with vimentin. The image was adapted from (Denielsson et al., 2018) and created with BioRender.com.

Single vimentin filaments are extremely extensible with the ability to be elongated up to at least 4.5-fold (Kraxner et al., 2021). Apart from the structural role of vimentin in regulating cell mechanics and stress resistance, more recent studies indicated that vimentin control gene regulation by interacting with signaling molecules and cell kinases (Paulin et al., 2022). Vimentin network is required to coordinate essential cellular functions such as mechanosensing, transduction, signaling pathways, motility, and inflammatory responses (Battaglia et al., 2018; Kraxner et al., 2021; Ostrowska-Podhorodecka et al., 2022; Paulin et al., 2022; van Beijnum et al.,2022). This network is especially important in physiologic and pathophysiologic functions related to wound healing and

tissue repair, fibrosis, angiogenesis, tumorigenesis, digestive diseases including Crohn's disease and colitis, inflammatory functions, and host response to infections (Wang & Stamenovic, 2002; Danielsson et al., 2018; Pandita et al., 2021; van Beijnum et al.,2022). Although previously mice lacking vimentin (Vim-/-mice) were reported to develop and reproduce normally without a clear phenotype, recently lack of vimentin is shown to be associated with phenotypes in various diseases (Table 3) (Ridge et al., 2022).

According to the Human Intermediate Filament database (http://www.interfil.org/), so far 119 distinct diseases have been associated with the IFs family (Omary, 2009; Sapra & Medalia, 2021). Regarding vimentin, a mutation in the human vimentin gene causes a rare multisystem disorder associated with premature aging (Cogné et al., 2020). Moreover, vimentin mutations have been identified in individuals diagnosed with cataracts (Mü Ller et al., 2009; Ma et al., 2016).

Recently, vimentin has emerged as a promising potential target in cancer therapy. Targeting vimentin organization and function using small molecules, antibodies, and compounds such as fluvastatin and simvastatin could reduce migration and invasion of highly motile metastatic cancer cells (Sjöqvist et al., 2021).

Table 3. Phenotypes associated with vimentin-null (Vim-/-) mice. The table is adapted from (Ridge et al., 2022).

Function	**Examples**
Cell proliferation, differentiation, migration, and tissue remodeling	• Impaired wound healing: embryonic cells, fibroblasts • Impaired differentiation and transdifferentiation: endothelial cells, hepatic cells, keratinocytes
Vascular Functions	• Impaired multilayer communication and structural haemostasis of arterial wall • Impaired cell rigidity and cell adhesion: circulating lymphocytes • Poor vascularization
Renal functions	• Impaired Na–glucose co-transportation and recovery
Metabolism and fat accumulation	• Impaired lipid accumulation • Impaired lipolysis
Viral and bacterial infections	• Impaired pathogen entry • Impaired viral replication

2.3.5. Vimentin and wound healing

Different studies showed that vimentin plays important role in the wound healing process (Ridge et al., 2022). Vim-/- cells display slow scab formation, defective fibroblast functions, impaired inflammatory and immune responses, and faulty angiogenesis which lead to impaired wound healing (Ridge et al., 2022). It has been shown that motile cells express higher levels of vimentin and lack of vimentin dramatically delays fibroblast migration and subsequent contraction of the wound (Battaglia et al., 2018). Besides the defects in fibroblast functions, lack of vimentin is associated with delayed appearance of myofibroblasts and defects in TGF-β signaling and the EMT process (Cheng et al., 2016). Furthermore, vimentin modulates the dynamics of cytoskeletal networks which generates sufficient force for wound contraction. It is well studied that the integrity of different cytoskeletal networks is dependent on one another. Specifically, the lack of structural support provided by vimentin leads to the impaired organization of non-intermediate filament cytoskeletal elements, reduced mechanical stability, and motility (Eckes et al., 2000; Challa & Stefanovic, 2011; Gladilin et al., 2014; Mendez et al., 2014; Boraas & Ahsan, 2016; Battaglia et al., 2018; Sharma et al., 2018; Hu et al., 2019; Patteson et al., 2019; Surolia et al., 2019; Vakhrusheva et al., 2019; Wilhelmsson et al., 2019; Schaedel et al., 2021; Ostrowska-Podhorodecka et al., 2022; Ridge et al., 2022).

2.4. Mechanical stress in wound healing

After the injury, cells in the wound site deal with serious changes in the mechanical forces which are produced by the injury itself or by the perturbation of epithelial sheet force balance. Human skin can convert environmental mechanical forces to biochemical signals which can affect wound healing outcomes. Previous studies showed that mechanical stress can alter the microenvironment of a healing wound by regulating cellular functions such as collagen fiber thickness, microvascular blood flow, inflammation, and signaling pathways (Cremers et al., 2015; Hastings & Shapiro, 2016; Fu et al., 2021; Kimura et al., 2021; Zhang et al., 2021; Jan et al., 2022; Monika et al., 2022; Sangwon et al., 2022; Xiaojie et al., 2022). Cells such as fibroblasts experience severe deformations during mechanical interactions and physiological processes such as EMT. In wound healing, fibroblasts undergo phenotypic changes during the EMT process and transition to myofibroblast (D'Urso & Kurniawan, 2020).

There are four main mechanical stresses that cells may experience during the wound healing process including tension, compression, shear, and osmotic

stress (Fig. 14) (Barnes et al., 2018). Although some mechanical forces such as stretching can be clinically used as wound healing mechanotherapy, increasing mechanical stress can directly be correlated with increasing dermal fibrosis and scar formation (Urschel & Williams, 1988).

Particularly, osmotic stress including hyper-osmotic and hypo-osmotic stresses can contribute to impaired wound healing. Generally, while hyper-osmolarity triggers cell shrinkage, hypo-osmolarity increases the cell volume (Brocker et al., 2012). It has been shown that signaling in cell proliferation, cell migration, and apoptosis is significantly affected by osmotic stress and changes in cell volume (Nielsen et al., 2008). Osmotic imbalance happens in different pathological conditions such as systemic hyperglycemia or prolonged edema in diabetes (Brocker et al., 2012).

During the wound healing process imbalance of osmotic conditions induces significant changes in signal transduction, gene expression, and metabolic activity of the cells which could cause impaired wound healing (Dascalu et al., 2000; Kruse et al., 2016; Jingi et al., 2017).

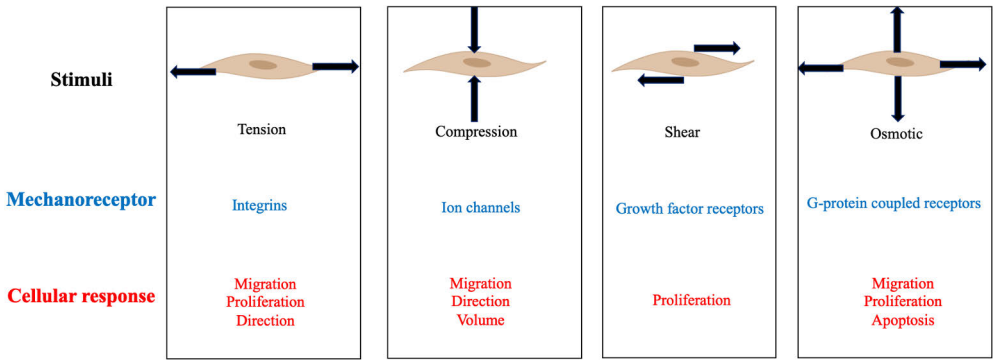

Figure 14. Schematic illustration of different types of cell mechanical forces and subsequent cellular responses. Four mechanical forces affect a cell's mechanical state: tension, compression, shear, and osmotic stress. Stimuli are transmitted to the cell via specific mechanoreceptors that trigger different cellular responses. The image was adapted from (Barnes et al., 2017).

2.4.1. Role of vimentin in cell mechanical stress

As we discussed in section 2.3, cytoskeletal proteins help cells to maintain mechanical integrity under large deformations. While actin and microtubule structures disassemble at moderate strains, IFs including vimentin are critical components to maintaining cellular strength, and stretchability at even higher strains (Eckes et al., 2000; Challa & Stefanovic, 2011; Gladilin et al., 2014; Mendez

et al., 2014; Boraas & Ahsan, 2016; Battaglia et al., 2018; Sharma et al., 2018; Hu et al., 2019; Patteson et al., 2019; Surolia et al., 2019; Vakhrusheva et al., 2019; Wilhelmsson et al., 2019; Schaedel et al., 2021; Ostrowska-Podhorodecka et al., 2022; Ridge et al., 2022). Vimentin is a hyper-elastic network that interacts with other cytoskeletal networks to disperse the local mechanical stress to a larger region in the cytoplasm and protects cells against mechanical damage (Mendez et al., 2014; Hu et al., 2019). It has been shown that osmotic stress regulates cytoskeletal protein expression and organization. (Mendez et al., 2014; Hu et al., 2019). Vimentin structural reorganization mediates resistance to osmotic stress by re-arrangement of different forms of vimentin with different molecular weights under varying conditions of osmolarity (Buchmaier et al., 2013).

Hypo-osmotic stress makes a rapid cytoplasmic extension by partially depolymerization and redistribution of vimentin in the cytoplasm. While lack of vimentin causes a dramatic decrease in cell survival rate during osmotic stress which indicates the critical contribution of vimentin to the defense mechanisms and osmotic endurance (Li et al., 2019).

2.4.2. Role of EVs in cell mechanical stress

In multicellular organisms, cells work together to perform complex physiological processes such as cell growth, differentiation, and response to environmental stress (Hedlund et al., 2011). It has been shown that besides acting as efficient intercellular communicators, EVs are powerful signal transductors to coordinate environmental stimuli. Environmental stressors such as thermal and oxidative stress (Hedlund et al., 2011), radiation, photodynamic treatment, and chemotherapy (Aubertin et al., 2016), low pH condition (Parolini et al., 2009), nutrient deficiency (Gao et al., 2016), anoxia, and hypoxia (King et al., 2012) and cytoskeletal rearrangements (Liu & Su, 2019) affect EVs release, distribution, compositions and function which eventually influence the physiological and pathological states of the cells (Qin, 2020). In this manner, EVs can be considered either as a stress modifier to restore the normal physiological condition or as a conveyer of stress to induce stress-driven conditions. For example, EVs are involved in mediating environmental stress in different reproductive cells by activating rescue molecular signals during stress (Gebremedhn et al., 2020; Chan et al., 2020). In cancer therapy, EVs are considered an emerging therapeutic nanoplatform (Ma et al., 2021) and can transmit survival messages in the tumor microenvironment to support resistance to therapy (Neill & Gilligan, 2019).

2.5. Extracellular vimentin

Vimentin was previously considered an intracellular protein with a structural role, however, recent evidence showed that vimentin can also be detected outside the cells (Patteson et al., 2020). The shorter and non-filamentous form of vimentin can be found on the cell surface or secreted in the extracellular environment either as a soluble form or as a vesicle-transported protein (Fig. 15). Phosphorylation of vimentin affects its intracellular localization and promotes its secretion into the extracellular space. This process is regulated by protein kinase C (PKC) and signaling by pro-and anti-inflammatory cytokines (Patteson et al., 2020). Extracellular vimentin has been shown to release during inflammation, cell activation, senescence, apoptosis, stress, and injury (Yu et al., 2018). Cell surface vimentin acts both as a receptor for different types of ligands such as carbohydrate chains and CD44 (Päll et al., 2011; Komura et al., 2012) and as a ligand for various receptors such as P-selectin (Lam et al., 2018). After vimentin is localized on the cell surface, the rod II domain of vimentin binds to GlcNAc-bearing polymers (Komura et al., 2012).

Upon injury, extracellular vimentin is distributed along the ECM substrate beneath the cells mostly on the surface of filopodia/lamellipodial (Yu et al., 2018). Extracellular vimentin promotes platelet adhesion (Da et al., 2014), angiogenesis (Lin et al., 2018), and fibroblast migration (Walker et al., 2018). In the wound healing process, extracellular vimentin could bind to the cell surface receptors such as CD44 and carbohydrate chains and transmits mechanotransduction signals to determine the cell fate (Päll et al., 2011; Komura et al., 2012). Activated macrophages secret extracellular vimentin as a proinflammatory factor and to remove the infection (Mor-Vaknin et al., 2003; Moisan & Girard, 2006). Furthermore, in spinal cord injury, vimentin is expressed by astrocytes and interacts with insulin-like growth factor 1 receptor to promote axonal growth and functional recovery (Jang et al., 2020; Shigyo et al., 2015). Transient senescence has been shown to enhance fibroblasts' activity and differentiation to prevent excessive fibrosis (Resnik et al., 2020; Wilkinson & Hardman, 2020). The expression of vimentin on the surface of fibroblasts could mediate the senescence process (Frescas et al., 2017). Interestingly, extracellular vimentin can act as a double-edged sword where it can promote the normal healing process or activate mesenchymal cells' transition to myofibroblasts and direct cell fate to fibrosis (Walker et al., 2018).

Different studies showed that EVs and specifically exosomes are one of the potential pools for extracellular vimentin which we have named it here "exosomal vimentin". Exosomal vimentin was reported to be detectable in

exosomes from different cell types. For example in colorectal cancer cells, exosomes act as a functional unit to efficiently transport functional vimentin to the target cells (Chen et al., 2016; Rahman et al., 2016).

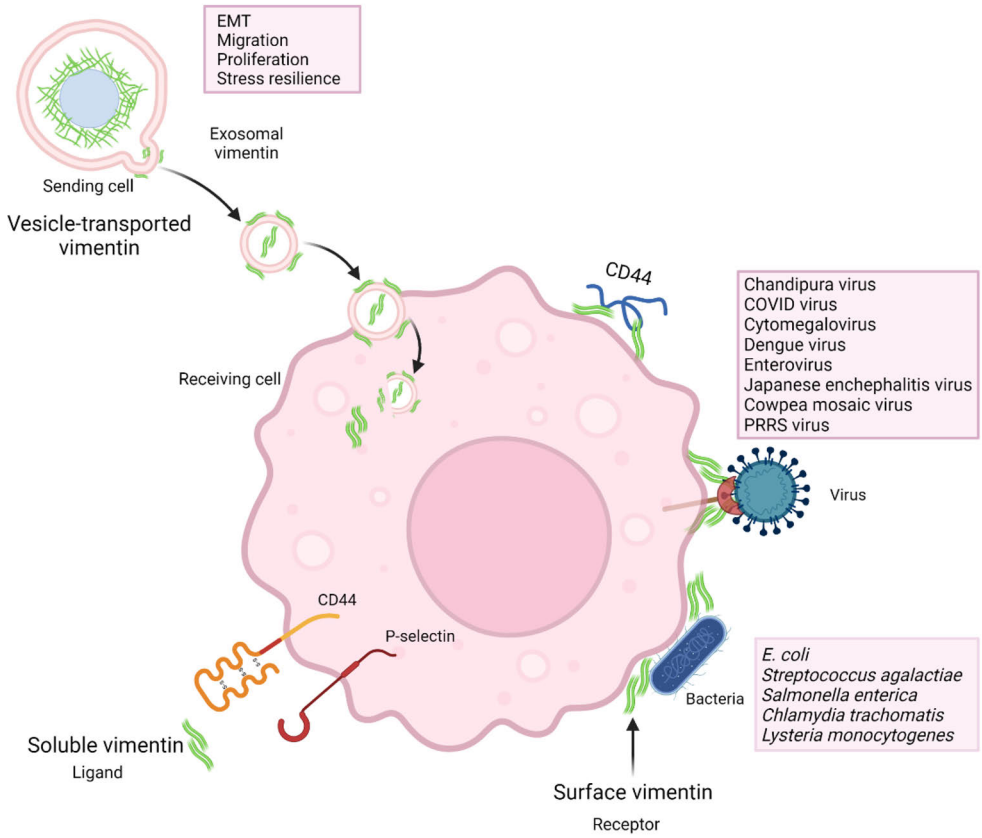

Figure 15. Extracellular vimentin pool and its roles. Extracellular vimentin can be found on the cell surface or secreted in the extracellular environment either as a soluble form or as a vesicle-transported protein. Extracellular vimentin mediates important biological functions such as cell proliferation, migration, senescence, inflammation, apoptosis, cell-cell interactions, and cell- pathogens interactions. The image was created with BioRender.com.

2.6. Mass production of extracellular vesicles

Due to the complexity of EVs cargo, small sizes, limited quantities, high variability in isolation and purification methods, and poor reproducibility, studying EVs is still challenging (Ramirez et al., 2018; Sharma et al., 2020). One

of the prerequisites for investigating the clinical application of EVs on an industrial scale is the reproducible large-scale production of EVs (Ramirez et al., 2018; Sharma et al., 2020). Hence consistent and effective mass production of EVs is one of the most important factors in their therapeutic application. It has been shown that the metabolic signature of EVs depends on the cell culture conditions of their parental cells (Palviainen et al., 2019) and preconditioning of cell culture can improve the production and clinical use of isolated EVs (Kim et al., 2018). This could happen using various methods such as genetic manipulation, exposure to hypoxia, increasing intracellular calcium, and treatment with bioactive molecules (Kim et al., 2018).

However, all of these modifications are approved in a traditional mono-layer culture which has a limited expansion, possibility of phenotypic changes, and loss of therapeutic activity during long-term passaging (Kim et al., 2018). Due to the significantly different microenvironment in tissue architecture, 2D cell monolayer by the traditional cell culture method is not capable to represent the physiology of *in vivo* 3D tissues or organs (Kim et al., 2018; Thippabhotla et al. 2019). It has been shown that 3D culture can improve paracrine function while producing more exosomes (Kim et al., 2018). According to previous studies, 3D spheroid culture stimulates the secretion of more *in vivo* like EVs and specifically increases exosome secretion from mesenchymal stem cells with higher therapeutic potential (Kim et al., 2018; Cha et al., 2018). Also, it has been reported that the production of EVs in bioreactors such as hollow-fiber bioreactors (Watson et al., 2016) (Patel et al., 2019) and CELLine Adhere 1000 (CLAD1000) flask (Mitchell et al., 2008; Palviainen et al., 2019) can maximize the EV yield in comparison to conventional cell cultures.

3. Outline and Key Aims of the Thesis

Vimentin is a cytoskeletal protein important for many cellular processes including wound healing. While its functions are usually related to vimentin as an intracellular protein, vimentin also can be exposed at the cell surface in an oligomeric form or secreted to the extracellular environment in soluble and vesicle-bound forms. Vimentin has been reported to be detectable in exosomes from different cell types. However, the role of exosomal vimentin in mediating wound healing had not been studied.

Prompted by previous findings underlying the involvement of extracellular vimentin in mediating tissue regeneration and wound healing, we hypothesized that extracellular vimentin in the vesicle form might have a role in wound healing. The key aim of my thesis was to elucidate the role of exosomal vimentin in promoting fibroblasts' functions in wound healing. Hence, I have isolated EVs from wild-type (WT) and Vim-/-ASCs and used human skin fibroblasts (HDFs) as a model to study the potential effect of exosomal vimentin on the healing process. Furthermore, I have studied the effect of osmotic stress on the vesiculation rate and specifically on exosome production, morphology, and function during wound healing. The results from *in vitro* studies were confirmed in *in vivo* models using mice skin injury. Moreover, I have developed a protocol to enhance exosome production using NFC in combination with the CELLine AD bioreactor.

Specifically, we aimed:

I. To investigate the existence and effect of exosomal vimentin from ASCs on fibroblasts' function in wound healing. (I)

II. To investigate how mechanical cell stresses such as osmotic stress affect exosome mediating wound healing. (II)

III. To develop an easy and efficient method to enhance EVs production from ASCs. (III)

4. Materials and Methods

The experimental procedures used in this thesis are presented in table 4. Detailed experimental procedures and used materials can be found in the original publications and the patent draft, at the end of the thesis.

Table 4. An overview of the experimental procedures used in this thesis.
*Experiments performed in collaboration with colleagues

Experimental procedure	**Study**
3D cell culture using nanofibrillar cellulose	III
BCA assay	I, II, III
Cell culture (APCs)	I, II, III
Cell culture (HDFs)	I, II, III
Cell culture using CELLine AD bioreactor	I, III
Cell-derived matrices (CDMs)	I, II
Cell proliferation by MTT assay	I, II, III
Cell transfection	I
Cell Apoptosis and Analysis	II
Directional cell migration assay	I, II
Dynamic Light Scattering (DLS)	I, II
Exosome isolation using ultracentrifugation	I, II, III
Exosome isolation using ultrafiltration	I
Exosome Labeling and Quantification	I, II, III
Fluorescent microscopy	I, II, III
Hematoxylin and eosin staining and quantification	I, II
Immunofluorescence staining (IF)	I, II, III
In vitro tracking (uptake assay)	I, II, III
Image analysis*	I, II, III
Mass spectrometry*	I
Mouse skin injury model and treatment	I, II
Nanoparticle Tracking Analysis (NTA)*	I, II, III
Osmotic stress induction	II
RNA isolation and qPCR analysis	I, II
Scratch closure assay	I, II
Statistical analysis	I, II, III
STED microscopy*	I
Transmission electron microscopy (TEM)	I, II, III
Western blot analysis (WB)	I, II, III

4.1. Cell culture

3T3L1 adipocyte progenitor cells (APCs) were used for exosome isolation. Human dermal fibroblasts were cultured as an *in vitro* model to study the effect of isolated exosomes (I, II, III) on fibroblasts' functions during wound healing. Cell culture media supplemented with EV depleted serum was used to isolate exosomes. APCs or preadipocytes are a small population of immature cells within the adipose tissue. The main function of APCs is to differentiate into fat cells (Pyrina et al., 2020).

While APCs commonly stand for antigen-presenting cells, during this thesis, APCs present adipocyte progenitor cells.

Table 5. List of the cell lines used in the thesis.

Cell line	Type	Study
3T3 L1 WT and Vim -/-	Adipocyte progenitors (APCs)	I, II, III
HDF WT and Vim -/-	Human dermal fibroblast	I, II, III

CELLine AD (Adhere) 1000 flask was used in some experiments to promote the production of EVs (I, III). For 3D culture in publication III, NFC called GrowDex® was used to form spheroids. According to the producer – UPM Biomedicals – GrowDex® is an animal-free, ready-to-use hydrogel that simulates and supports cell growth and differentiation.

4.2. Exosome isolation and labeling (I, II, III)

APCs-derived exosomes (APC-Exos) were isolated using differential ultracentrifugation (I, II, III). However, in study I, besides differential ultracentrifugation, the ultrafiltration method was used to maximize capturing of exosomal vimentin especially those that are incorporated into the surface of exosomes.

In study II, to investigate the role of exosomal vimentin in mediating osmotic stress conditions, exosomes were isolated from six conditioned media which are summarized in table 5.

Table 6. Conditioned media are used in the studies of this thesis. H^-: hypo-osmotic stressed, H^+: hyper-osmotic stressed.

	Cell source	Abbreviation
1	Exosomes from wild-type adipocyte progenitor cells	WT-Exo
2	Exosomes from vimentin knockout adipocyte progenitor cells	Vim-/-Exo
3	Exosomes from wild-type hypo-osmotic stressed adipocyte progenitor cells	WT-H^-Exo
4	Exosomes from vimentin knockout hypo-osmotic stressed adipocyte progenitor cells	Vim-/-H^-Exo
5	Exosomes from wild-type hyper- osmotic stressed adipocyte progenitor cells	WT-H^+Exo
6	Exosomes from vimentin knockout hyper-osmotic stressed adipocyte progenitor cells	Vim-/-H^+Exo

4.3. Exosomes characterization (I, II, III)

After exosome isolation and before any further analysis, the protein content of isolated exosomes was measured using the Pierce BCA protein assay kit. The optimal dose of APC-Exos to affect the proliferation and migration of HDFs was tested in a wound scratch assay. Exosomes were characterized using western blot (WB), NTA, and TEM microscopy (I, II, III). In study I, DLS analysis was used to measure the size of the exosomes, and STED microscopy was used to confirm the incorporation of vimentin into the isolated exosomes. In studies II and III, fluorescent microscopy was used to measure the number of fluorescently labeled exosomes.

4.4. Protein expression (I, II, III)

In this thesis, WB was used to detect exosomal markers (CD9, CD63, CD 81, and Hsp70), apoptosis marker (PARP-1), vimentin, and ECM proteins such as collagen I. The architecture of Cell-derived matrices (CDMs) was visualized by immunofluorescence staining for collagen I and fibronectin. Vimentin and CD9 antibodies were used in ImmunoEM to characterize isolated exosomes. A vimentin antibody was also used in STED microscopy to detect the localization of vimentin and fluorescently labeled exosomes. The list of the antibodies used in this thesis is presented in table 6.

Table 7. Primary antibodies are used in the studies of this thesis and their applications. WB: western blot, IF: immunofluorescence, ImmunoEM: immune-electron microscopy.

Antibody	Company	Application	Study
β-actin	Cell Signaling	WB	II
CD9	Novus biological	ImmunoEM	II
CD9	System Biosciences	WB	I, II, III
CD63	System Biosciences	WB	I, II
CD81	System Biosciences	WB	I, II, III
Collagen I	Novus	IF	I, II
Fibronectin	Sigma-Aldrich	IF	I
GAPDH	System Biosciences	WB	I, II
Hsp70	System Biosciences	WB	I, II, III
PARP-1 (clone F2)	SantaCruz	WB	II
Vimentin (clone O91D3)	Biolegend	WB	I, II
Vimentin (clone RV203)	Novus	ImmunoEM	II

4.5. Mouse skin injury model (I, II)

For *in vivo* wound healing model, 5–8 weeks old male mice were anesthetized, wounded, and then treated with isolated exosomes. In study I, mice bare skin was treated with WT and Vim-/-Exos, and in study II, treatments were done using WT, Vim-/-, WT-H^+, WT-H^-, Vim-/- H^+, and Vim-/-H^-Exos. For histology analysis, mice were sacrificed by cervical dislocation and their wound tissue samples were biopsied, fixed, and stained with hematoxylin and eosin. Furthermore, total RNA from skin tissue and spleen was isolated and gene expression of the following genes was measured by quantitative polymerase chain reaction (qPCR).

Table 8. List of genes and their primers for qPCR analysis.

Gene	Forward Primer Sequence5′ →3′	Reverse Primer Sequence 5′ →3′	Study
Mouse-β-Actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT	I, II
Mouse-Col-I	GAGCGGAGAGTACTGGATCG	TACTCGAACGGGAATCCATC	I
Mouse-Granzyme B	TCGACCCTACATGGCCTTAC	TGGGGAATGCATTTTACCAT	II
Mouse-IL-6	GTTCTCTGGGAAATCGTGGA	TGTACTCCAGGTAGCTATGG	I
Mouse-IL-10	CGGGAAGACAATAACTGCACCC	CGGTTAGCAGTATGTTGTCCAGC	I
Mouse-IL-12	CAGCATGTGTCAATCACGCTAC	TGTGGTCTTCAGCAGGTTTC	II
Mouse-TGFβ1	CTCCCGTGGCTTCTAGTGC	GCCTTAGTTTGGACAGGATCTG	I
Mouse-TNF-α	TCTCATCAGTTCTATGGCCC	GGGAGTAGACAAGGTACAAC	I, II

5. Results and Discussion

5.1. Exosomal vimentin accelerates wound healing by affecting fibroblasts' functions

5.1.1. WT-APC-Exos enhance the proliferation of HDFs in vitro (I, II)

ASC-Exos have been previously shown to carry various bioactive factors that biologically benefit fibroblasts' functions in wound healing. ASC-Exos restore the epidermal barrier and enhance skin elasticity by downregulating the expression of inflammatory cytokines such as interleukins (IL-1β, IL-4, IL-5, IL-6, IL-8, IL-13), TNF-α, and interferon-gamma (IFN-γ) and upregulating TGF-β, MMP-1, ceramides, and procollagen type I (Hassan, Greiser, and Wang 2014) (Zgheib et al., 2014). Furthermore, ASC-Exos enhance the re-epithelization by reducing the production of reactive oxygen species (ROS) and oxidative stress-related proteins such as NADPH oxidase 1/4 (NOX1/4) and increasing the expression of MMP-9 and vascular endothelial growth factor (VEGF). Moreover, ASC-Exos have been reported to increase tissue thickness by enhancing tube formation of vascular endothelial cells and decreasing infiltration of inflammatory cells and apoptosis (Xiong et al. 2020).

Basic fibroblast growth factor (bFGF) is a cytokine that is clinically widely used in accelerating the outcome of wound healing. The bFGF enhances the quality of scar and regeneration by inducing fibroblasts proliferation, angiogenesis, and recruiting leukocytes to the inflammation sites (Akita et al., 2013). During our study, bFGF was used as a positive control to enhance HDFs' behavior toward healing such as proliferation and migration.

In this thesis, isolated exosomes were characterized for their morphology, size, and expression of the surface markers by different methods (I, II, and III). To find the optimal concentration of APC-Exos, three different doses (10, 100, and 200 μg/ml) were tested in a wound scratch assay. The results showed that 100 μg/ml of WT-APC-Exo was the optimal concentration to enhance wound closure (I, Fig. 2 (a-d)). In study I, the effect of WT and Vim-/-APC-Exos on HDFs' proliferation was measured by MTT assay and microscopy images (I, Fig. 3). The results showed that while both Exos promote HDFs' proliferation compared to the negative control (DM), a higher proliferation rate was observed for treatment with WT-APC-Exos. In study II, when we measured the effect of WT and Vim-/-APC-Exos on HDFs proliferation in osmotic stress conditions, the same results were obtained (II, Fig. 3 (c)).

5.1.2. WT-APC-Exos enhance the migration of HDFs in vitro (I, II)

Exosomes have been previously reported to promote cell directional migration in two ways: firstly, exosomes as a carrier of ECM components, simulate extracellular receptor signaling and enhance matrix attachment and cell migration. Secondly, exosomes carry a cargo of several necessary molecules that promote cell motility including proteinases, chemokines, and growth factors (Sung et al., 2015). Also, it has been frequently reported that vimentin plays a crucial role in the determination of fibroblast directional migration (Margiotta & Bucci, 2016).

In Study I, our results confirmed that WT-APC-Exos significantly drive directional cell motility towards the same y-coordinate in a linear path to close the wound. Interestingly, despite an increase in cell velocity, Vim-/-APC-Exo imposed a directionless path of a zigzag' pattern to close the wound (I, Fig. 2 (e-g)). Furthermore, in the line with these results, in study II, we observed a random cell orientation for non-treated (controls) and treated HDFs with Vim-/-Exos, while HDFs treated with WT-Exos were aligned in a certain direction (II, Fig.5 (a and b)).

5.1.3. WT-APC-Exos promote ECM production by HDFs in vitro (I, II)

ECM components such as collagen and fibronectin are critical for wound healing. Vim-/- fibroblasts have been shown a reduction in ECM production which leads to delayed wound healing (Cheng et al., 2016). Furthermore, exosomes have been reported to promote the healing process by regulating ECM remodeling (Wang et al., 2017).

CDMs are decellularized extracellular matrices that recapitulate the composition and organization of native ECM microenvironments. CDMs could provide a better physiologically relevant alternative to studying in vivo-like cell behavior in an *in vitro* model (Fitzpatricka, 2008). CDMs consist of a complex mixture of fibrillar proteins, matrix macromolecules, and growth factors (Kaukonen et al., 2017). Hence, in this thesis to study ECM production, CDMs secreted by HDFs were used to mimic *in vivo* microenvironment.

In studies I and II, we observed that WT-APC-Exo could significantly enhance the production of CDMs by HDFs when compared to Vim-/-APC-Exo (I, Fig. 5 and II, Fig. 5 (c and d)). These results could suggest that exosomal vimentin as one of the extracellular vimentin pools plays a key role in the reconstruction and organization of the ECM components.

5.1.4. WT-APC-Exos promote wound healing in vivo (I)

As mentioned earlier, growth factors and cytokines such as ILs are essential mediators in regulating inflammatory cells and the healing process. For example, the downregulation of IL-6, IL-8, and TNFα and upregulation of anti-inflammatory cytokines such as IL-10 and TGF-β are crucial for successful wound healing (Zgheib et al., 2014). Impaired wound healing has been linked to increased levels of TNF-α and IL-6 and decreased levels of anti-inflammatory IL-10 (Xu et al., 2013). TGF-β is a cytokine with powerful anti-inflammatory functions that is crucial for epidermal haemostasis, re-epithelialization, and angiogenesis (Ramirez et al., 2014). TNFα is a proinflammatory cytokine that quickly releases and initiates inflammation at wound sites. IL-6 is produced at the site of inflammation and mediates the transition from acute to chronic inflammation (Gabay, 2006).

Our results from *in vivo* experiments confirmed the *in vitro* data and showed that wounds treated with WT-APC-Exos significantly healed faster compared to Vim-/-APC-Exos and control groups (I, Fig. 6 (a and b)). Moreover, histological analysis revealed that WT-APC-Exo treatments reduce inflammation and immune cell infiltration by downregulating pro-inflammatory cytokines such as IL-6 and TNF-α and up-regulating anti-inflammatory cytokines such as IL-10 (I, Fig. 6 (c)). Furthermore, RT-qPCR analysis showed that TGFβ and collagen I were significantly higher in the WT-APC-Exos group while IL-6 and TNFα were lower (I, Fig. 6 (d-g)).

5.1.5. Vimentin carried by exosomes is internalized by HDFs (I)

Previously, it has been shown that the biogenesis, secretion, and uptake of EVs require the remodeling of cytoskeletal proteins (Mulcahy et al., 2014; Margiotta & Bucci, 2016). Furthermore, surface interactions of EVs with their recipient cells are regulated by cytoskeleton components which participate in the fusion and transport of intracellular membranes (Théry et al., 2002; Buzás et al., 2018).

Intracellular vimentin has been reported to attach to the nucleus, endoplasmic reticulum, and mitochondria (Challa & Stefanovic, 2011). Upon injury, vimentin can be localized to the cell surface in an oligomeric form or released to the extracellular space in soluble and vesicle-bound (Yu et al., 2018). According to previous studies, cytoskeleton proteins including vimentin are detectable in extracellular vesicles and specifically in exosomes (Kumari et al., 2015; Rahman et al., 2016; Dozio & Sanchez, 2017; Tucher et al., 2018; Adolf et al., 2019).

To confirm the observed effects of exosomal vimentin on fibroblasts' proliferation, migration, and ECM production, both WT and Vim-/-APCs were transfected with pEGFP-VIM plasmid. Our results confirmed the incorporation of pEGFP-VIM in both cell lines as well as their isolated exosomes. Figure 15 (a) shows the successful transfection of pEGFP-VIM in Vim-/- APCs. Figure 15 (b) demonstrates the uptake of exosomes isolated from transfected Vim-/- APCs by HDFs.

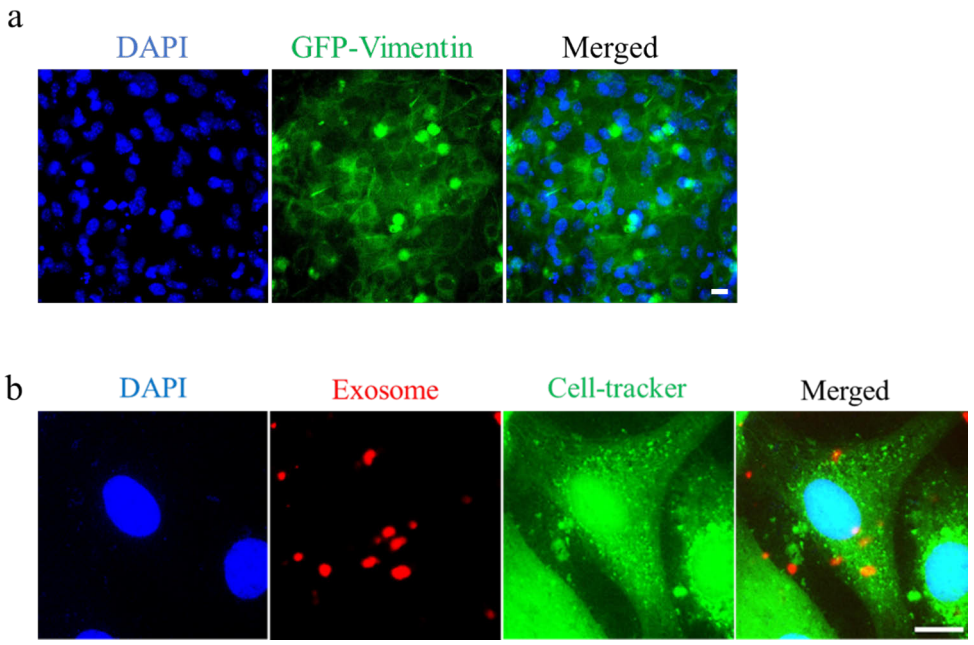

Figure 16. (a) Representative images of successful transfection of Vim−/-APCs with pEGFP-VIM plasmid 24-hour post-treatment. (b) Representative images of internalization of exosomes isolated from transfected Vim-/- APCs with pEGFP-VIM plasmid by HDFs 24-hour post-treatment. Scale bar: 10 µm.

Furthermore, our result from the exosome uptake assay showed that while both WT and Vim-/- Exos were taken up by HDFs, a higher uptake percentage was achieved for WT-APC-Exo (I, Fig. 5 (a-d)). This data suggest that vimentin might play a role in exosome uptake and internalization. We then confirmed the incorporation of vimentin into the WT-APC-Exos using STED microscopy (I, Fig. 5 (e)) and mass spectrometry (I, table 2). The results of both experiments confirmed that vimentin is incorporated into the WT-APC-Exos but not Vim-/-APC-Exos. Specifically, the results from STED microscopy showed that vimentin

could be found either on the surface or inside the exosomes. Nevertheless, further experiments are needed to clarify the packing process and structural organization of exosomal vimentin.

5.2. Exosomal vimentin protects fibroblasts against osmotic stress

5.2.1. WT-APCs tolerate osmotic stress better than Vim−/−APCs (II)

Osmotic stress cause changes in cell volume, cell viability, and proliferation by affecting different signaling pathways (Eduardsen et al., 2011; Sachs & Sivaselvan, 2015; Chen & Li, 2017). In osmotic stress conditions, vimentin forms a sponge-like interior cage to the membrane that distributes osmotic stress throughout the cell volume (Buchmaier et al., 2013; Li et al., 2019).

Our findings from culturing WT and Vim-/- APCs under osmotic stress conditions indicated that hypo-osmotic stress media increase and hyper-osmotic stress media decrease the cell volume, and these changes are higher in Vim-/- APCs (II, Fig.1). Furthermore, these results showed that WT-APCs can tolerate osmotic stress better than Vim-/-APCs and lack of vimentin makes cells significantly vulnerable to environmental stress, which could point out the role of vimentin in supporting cells against osmotic stress.

5.2.2. Osmotic stress increases exosome size and production (II)

The composition of exosome cargo is significantly influenced by environmental changes that affect the outcome of communication between the exosome-producer and the recipient cells (Villarroya-Beltri et al., 2014). Besides the role of exosomes in cell-cell communication, exosomes are considered an alternative way to eliminate waste products. Exosomes containing cell waste materials have been shown to communicate with neighboring cells about intracellular stress. In this manner, the larger size and number of exosomes could be explained by two mechanisms: firstly as an efficient route to dispose of cellular waste, and secondly as a communicator carrier of signaling, toxic, and regulatory molecules to modify other cells' functions (Fader et al., 2008; de Jong et al., 2012; Soria et al., 2017).

Our results from DLS, NTA, WB, TEM, and immunofluorescence microscopy showed that osmotic stress increases the size and the number of secreted exosomes by both WT and Vim-/-APCs (II, Fig. 2).

5.2.3. WT-Exos enhance proliferation and prevent apoptosis of osmotic-stressed HDFs (II)

Autophagy is an intracellular vesicular-related process that promotes cell survival and maintains intracellular haemostasis during stress conditions. Autophagy can happen before the programmed cell death process called apoptosis (Baixauli et al., 2014; Chen et al., 2018; Salimi et al., 2020). Apoptosis is mediated by proteolytic enzymes called caspases. The caspase cascade including caspase-3 and caspase-7 is responsible for executing cell death by proteolytic cleavages of several substrates such as structural components of the cytoskeleton and nucleus, as well as numerous proteins involved in signaling pathways (Lakhani et al., 2006). Poly ADP (adenosine diphosphate)-ribose polymerase (PARP-1) is a chromatin-associated protein that is involved in maintaining DNA stability and repair. PARP-1 is one of the critical substrates that is cleaved by caspases which could be considered an indicator of functional caspase activation (Puig et al., 2001). To study apoptosis, we measured the expression of cleaved caspase-3, cleaved caspase-7 as well as cleaved PARP-1 in cells undergoing apoptosis.

Exosomes have been previously shown as cellular waste disposal compartments when the transport through the degradative or lysosomal pathway is hindered due to stress (Fader et al., 2008). It has been reported that in stress and pathological conditions exosomal and cell death pathways such as autophagy are cross regulated. MSC-Exos have been shown to protect cells against stress-induced apoptosis (Z. Liu et al., 2019).

Vimentin is a highly stable and stress-resistant cytoskeleton protein. Vimentin has been shown to mediate autophagy by regulating organelles distribution (Id et al., 2019). As mentioned earlier, upon injury, vimentin releases into the extracellular space and binds to the cell surface of repair-modulating cells (Buchmaier et al., 2013; Li et al., 2019). Vimentin-containing vesicles are one of the potential sources of extracellular vimentin pool (Shigyo & Tohda, 2016; Patteson et al., 2020).

Prompted by our previous findings underlying the involvement of exosomal vimentin in promoting cell proliferation, migration, and ECM production, we explored whether exosomal vimentin plays a role in cell resistance to osmotic stress and cell protection against apoptosis. Our results showed that the co-culture of osmotic-stressed HDFs with WT-Exos considerably suppressed both hypo and hyper osmotic-induced apoptosis compared to non-treated osmotic-stressed HDFs (II, Fig. 4 (a, b, e, f, and h)).

5.2.4. Osmotic-stress-induced exosomes influence HDFs proliferation and apoptosis (II)

As we discussed in section 5.2.2, the composition, biogenesis, and secretion of exosomes are greatly affected by cellular stress conditions (Parolini et al., 2009; Hedlund et al., 2011; King et al., 2012; Aubertin et al., 2016 ; Liu & Su, 2019). We have also separately discussed the importance of exosomes and vimentin in protecting cells against apoptosis in section 5.2.3.

We then studied the effect of exosomes from osmotic-stressed APCs on HDFs' proliferation and apoptosis. HDFs were co-cultured with WT-H-Exos, Vim-/-H-Exos, WT-H+Exos, and Vim-/-H+ Exos, and proliferation and apoptosis rates were measured using WB and microscopy. It appeared that HDFs' proliferation was slowed down (II, Fig. 3 (d)) and the apoptosis rate was increased after treatment of normal HDFs with hypo and hyper osmotic-stressed exosomes (II, Fig. 4 (c, d, e, g and i)). Taken together, our data showed that WT-Exos significantly suppressed osmotic-induced apoptosis, whereas exosomes from osmotic-stressed APCs can induce apoptosis. This data indicated exosomes either as stress modifiers to maintain the osmotic balance or as a conveyer of stress to induce osmotic stress-driven conditions.

5.2.5. WT-APC-Exos promote wound healing in an osmotic-stressed in vivo model (II)

To confirm our results obtained from *in vitro* experiments, we tested the involvement of exosomal vimentin in wound healing in an *in vivo* osmotic-stressed mouse model. The results demonstrated that wounds treated with WT-APC-Exos healed faster with a minimum scar size than mice treated with Vim-/-APC-Exos (II, Fig. 6 (a-c)). IL-12 is an endogenous inhibitor of re-vascularization and our RT-qPCR analysis showed that IL-12 was significantly higher in the Vim-/- Exo group or control group when compared to the WT-APC-Exos group (II, Fig. 6 d). Our results may suggest that WT-APC-Exos can modify osmotic stress-induced apoptosis in fibroblasts while exosomal vimentin plays a vital role in this process.

5.2.6. WT-APC-Exos affect collagen fiber orientation and promote ECM production by osmotic stressed HDFs (II)

As we previously discussed in section 5.1.2, exosomes can mediate efficient cell directional migration by carrying ECM components and necessary molecules for cell motility such as proteinases, chemokines, and growth factors (Sung et al.,

2015). Moreover, we discussed that vimentin is crucial in the determination of fibroblast directional migration (Margiotta & Bucci, 2016).

Encouraged by our results from section 5.1.2, we studied the effect of WT and Vim-/-APC-Exos on the orientation of ECM fibers using CDMs secreted by osmotic stressed HDFs. Our results showed that the directionality of collagen fibers was similar to the direction of their original cells (II, Fig. 6 (a and b)). Furthermore, the fibers from CDMs secreted by WT-Exos treated HDFs were aligned in a certain direction. But there was a random fiber orientation for non-treated HDFs (controls) and treated HDFs with Vim-/-Exos, (II, Fig. 6 (c-f)).

Our results from studies I and II showed that exosomal vimentin played a significant role in mediating wound healing by affecting fibroblasts' functions. Besides the mechanical and structural functions of vimentin in regulating cell mechanics, vimentin has non-mechanical functions in both filamentous and non-filamentous forms. Vimentin controls important cellular functions such as cancer, traumatic tissue injury, bacterial or viral infection, rheumatoid arthritis, multiple sclerosis, aging and senescence, innate and adaptive immune responses, and thrombosis via interactions with other cytoplasmic intermediate filaments and cellular signaling molecules (Messica et al., 2017; Hu et al., 2019; Patteson et al., 2019; Kraxner et al., 2021; Schaedel et al., 2021; Ostrowska-Podhorodecka et al., 2022; Paulin et al., 2022; Ridge et al., 2022). Our results from HDFs treated with WT and Vim-/-APCs-Exos showed that lack of extracellular vimentin in a vesicle form could adversely affect fibroblasts' function and delay the healing process. In our experimental setup, lack of vimentin was the only difference between treatments. However, the observed effect from Vim-/-APCS-Exos treatments might be due to the loss of other cellular and molecular interactions mediated by vimentin such as interactions with other cytoskeleton proteins and signaling molecules. Furthermore, comparing HDfs treated with both WT and Vim-/-Exos with negative control (depleted media after exosome isolation) demonstrated that regardless of having exosomal vimentin, both Exos could significantly enhance fibroblasts' functions. These data indicate the importance of exosomal cargo such as growth factors, lipids, DNA, and RNA in optimizing the functions of recipient cells.

5.3. Efficient production and enhanced delivery of exosome using nanofibrillar cellulose- bioreactor cell culture method

5.3.1. 3D cell culture using nanofibrillar cellulose stimulates the secretion of in vivo-like exosomes (III)

It has been shown that MSCs in the 3D spheroid form released more IL-1 and proangiogenic cytokines such as VEGF, bFGF, and angiogenin (Kim et al., 2018). 3D spheroid culture has been previously shown to enhance paracrine function and stimulate the secretion of *in vivo*-like extracellular vesicles compared to conventional monolayer culture (2D) (Kim et al., 2018; Cha et al., 2018). The increased secretion of MSC-Exos is caused by the changes in the morphology of spheroids to non-adherent round shapes (Kim et al., 2018).

In study III, we first optimized the optimal concentration of NFC for both HDFs and APCs. Our results showed that 0.2% and 0.4% NFC in cell culture media were the optimal concentrations to obtain spheroids by APCs and HDFs, respectively (III, Fig. 1). We then measured the production of APC-Exos using NFC 3D cell culture by NTA, WB, and microscopy. Our data showed that this cell culture method stimulates the secretion of exosomes by APCs spheroids compared to 2D culture (III, Fig. 2, 3, and 4). We then tested the functionality of APCs-Exo isolated from 2D and 3D cultures in an uptake assay. To do that four uptake experiments were performed including 2D HDFs + 2D Exo, 2D HDFs + 3D Exo, 3D HDFs + 2D Exo and 3D HDFs + 3D Exo. The results showed that 3D HDFs treated with APC-Exos from 3D culture present the highest uptake efficiency (III, Fig. 5).

5.3.2. Cell culture using a Bioreactor in combination with nanofibrillar cellulose enhances the production and delivery of exosomes (III)

Studies showed that the production of EVs in bioreactors such CELLine Adhere 1000 (CLAD1000) flask significantly enhances the cell culture period and consequently the EV yield in comparison to conventional cell cultures (Mitchell et al., 2008; Palviainen et al., 2019; Patel et al., 2019).

Encouraging by our results from the previous section, we tested if the combination of Bioreactor and NFC could enhance exosome production even more. Since the cell compartment in CELLine Adhere 1000 flask is designed for adherent cells, we first optimized the surface of the bioreactor for 3D culture

using 0.5% agarose. Then APCs spheroids were cultured in the bioreactor. Proliferation and exosome production were measured after 5, 7, 10, and 14 days of culture. Our results showed that compared to 2D culture, 3D culture using NFC, and bioreactor culture, by this method, cell culture can continue for a longer time (up to 30 days). Furthermore, the results showed that this method compared to the other mentioned methods promotes cell proliferation (III, Fig. 9) and exosome production (III, Fig. 10).

6. Conclusions

Altogether, our findings indicated that exosomal vimentin by promoting the function of fibroblasts contributes to the wound healing process. Furthermore, the results of this study showed that in osmotic stress, exosomal vimentin protects cells against stress-induced apoptosis (Fig. 15). This observation raises the possibility that cytoskeleton disruption could have an impact on exosome transportation, internalization, and, finally, cell communication mediated by exosomes. However, future investigations are necessary to investigate the mechanisms behind the packaging of vimentin into the exosomes, the secretion and organization of exosomal vimentin, its delivery to recipient cells, and related signaling pathways and PTMs. Advances in exosome isolation and purification techniques, imaging, and single-cell exosomics may help to characterize exosomal vimentin further. Such studies could significantly broaden our understanding of exosomes as novel cell-free agents in mediating wound healing and cellular stress.

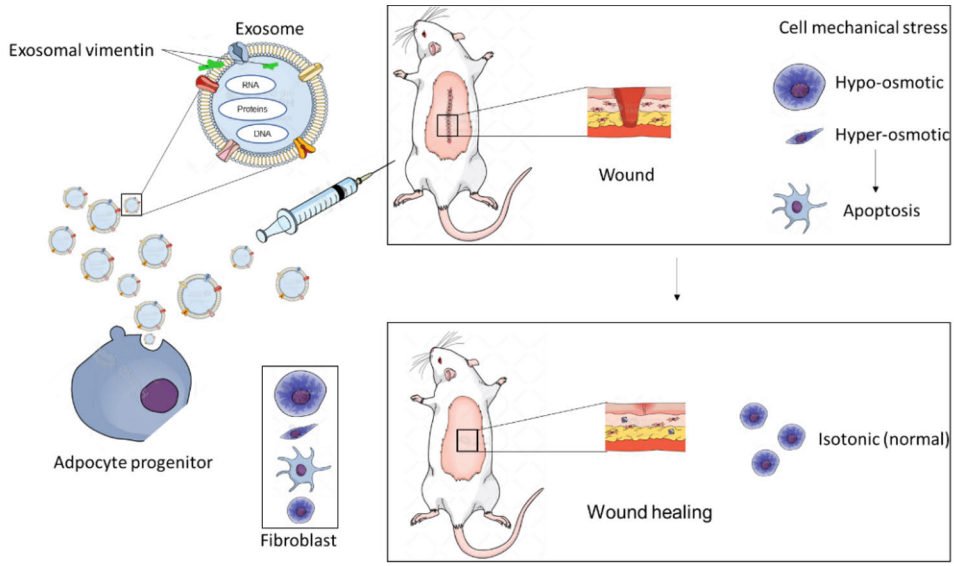

Figure 17. Schematic illustration of the role of exosomal vimentin in mediating wound healing in osmotic stress fibroblasts. The image was created with BioRender.com.

7. Acknowledgments

First and foremost, I am extremely grateful to my supervisors, Professor John Eriksson and associate professor Fang Cheng (Rose) for their invaluable advice, continuous support, and patience during my Ph.D. study. Their immense knowledge and plentiful experience have encouraged me in all the time of my academic research and daily life. Especially, I am deeply indebted to Prof. Eriksson for all his support during my study. John, you have been an amazing mentor and a great supporter through challenges during these years. Your kindness, humility, and great leadership skills have taught me a lot during these years. You have provided me with great opportunities, and you've given me the chance to grow. I'm truly grateful to you. Rose, I would like to thank you for taking me as your graduate student and giving me the opportunity to grow and improve my academic skills.

I would also like to express my deepest gratitude to Prof. Annika Meinander for her support during my study and especially my research visit to Harvard Medical School. This endeavor would not have been possible without your help. Besides my advisor, I would like to thank the rest of my thesis committee: Prof. Tapani Viitala, associate professors Diana Toivola, and Guillaume Jacquemet who accepted to be my committee even with short notice. Professors. Seppo Vainio and Pia Siljander are deeply acknowledged for accepting to be pre-examiners for this thesis. Your constructive comments and suggestions have undoubtedly improved my thesis. Especially Professor Vainio for the great support and advice on my future academic career at Harvard Medica School. I greatly thank Associate Professor Alison Patteson for accepting to be the opponent in the public defense of this dissertation.

I would like to extend my sincere thanks to all my office mates and the members of Eriksson's lab. It is their kind help and support that have made my study and life in Finland a wonderful time. Especially I would like to thank Leila Rato, Elnaz Fazeli, Michael Silva, Arun Venu, Preethy Paul, Alia Joko, Ponnuswamy Mohanasundaram, Mayank Modi, Emilia Holm, Giulia Sultana, Navid Delshad, Peiru Yang and Silvia Gramolelli. Leila Rato, thank you very much you have been a great friend and office mate.

I also would like to thank the Doctoral Program in the faculty of science and engineering at Åbo Akademi University for supporting my research and my travels during these years. Also, I express my gratitude to the Ella and Georg Ehrnrooth Foundation, Sigrid Jusélius Foundation, Harry Elvings legat, and UPM Biofore company for their financial support at times during these years.

I would like to express my sincere gratitude to Professor Donald Ingber and Associate Professor Sean Hall at Harvard Medical School/ Wyss Institute for welcoming me to their research group as part of my Ph.D. study.

Getting through my dissertation required more than academic support and I could not have undertaken this journey without my amazing friends. Living abroad far from family and friends, friends become family and I would like to express my gratitude to all my friends in Turku who made being far from family bearable. Dr. Solmaz Amini, thank you very much for all the Saturday brunches, long chats, loud laughs, and sympathy tears. You have been always a supportive and encouraging friend. Dr. Ali Daraei, thank you very much for being such an amazing friend. You have been my best friend for years and I never forget all your support and kindness. Sadaf Fazeli thanks for all the lunches, chitchats, and laughs. Behnoush Gustafsson thanks for your kindness and support. Elnaz Fazeli thanks for being such an amazing and supportive friend. Dr. Mark Percival, thanks for all your positive vibes and encouraging words. You have been an amazing friend. Professor Martin Cloonan, thank you very much for your kind advice and encouraging words. I am extremely grateful for our friendly chats and your support in my academic and business endeavors. From the bottom of my heart, I would like to say a big thank you to Dr. Mehrbod Mohammadian. Mehrbod, Thank you for your endless amount of support, love, and encouragement. I have always appreciated how you stay calm even in the most challenging and stressful times in my life. For all the moments that you hold my hands and say you will be alright! Thank you!

Last but not least, I would like to express my heartfelt gratitude to my parents, sisters, my brother, and my niece for their continued love and support. I am forever grateful for my caring, patient, and supportive family. This accomplishment would not have been possible without them. My beautiful Mom, you are my best friend in the whole world. You have taught me how to be disciplined, dream big, and never give up. I love you. My amazing dad, I am so fortunate to have a dad like you. You have always been supportive and encouraging. You taught me how to live and be strong. You are my rock. You have always been my inspiration on this path from the very beginning. I love you so much. Moji, Khatereh, and Matin, you are such amazing siblings, you are my best friends, and I am so proud to be your sister. I love you. Sogand, my beautiful and smart niece, I kept your lovely note with your adorable language as my laptop background when you wrote it to me with your tiny, tiny hands how much you missed me. I love you!

Finally, I would like to thank all those people who made this thesis possible as an unforgettable experience in my life. It was a pleasant task to express my

thanks to all those who contributed in many ways to the success of this study and also made it a remarkable experience for me.

Boston, October 2022
Sepideh Parvanian

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ISBN 978-952-12-4238-0