Dissertations in Health Sciences

UMA THANIGAI ARASU

HYALURONAN-COATED EXTRACELLULAR VESICLES

Regulation of their secretion and interactions with the target cells
HYALURONAN-COATED EXTRACELLULAR VESICLES

- REGULATION OF THEIR SECRETION AND INTERACTION WITH THEIR TARGET CELLS
Uma Thanigai Arasu

HYALURONAN-COATED EXTRACELLULAR VESICLES

- REGULATION OF THEIR SECRETION AND INTERACTION WITH THEIR TARGET CELLS

Publications of the University of Eastern Finland
Dissertations in Health Sciences
No 557

University of Eastern Finland
Joensuu/Kuopio
2020
Author’s address: Institute of Biomedicine/School of Medicine
University of Eastern Finland
KUOPIO
FINLAND

Doctoral programme: Doctoral programme in molecular medicine

Supervisors: Docent Kirsi Rilla, Ph.D.
Institute of Biomedicine/School of Medicine
University of Eastern Finland
KUOPIO
FINLAND

Docent Sanna Oikari, Ph.D.
Institute of Biomedicine/School of Medicine
University of Eastern Finland
KUOPIO
FINLAND

Reviewers: Professor Johanna Ivaska, Ph.D.
Turku Biocenter
University of Turku
TURKU
FINLAND

Adj. Professor Aki Manninen, Ph.D.
Department of Biochemistry and Molecular Medicine
University of Oulu
OULU
FINLAND

Opponent: Professor Mattias Belting, M.D, Ph.D.
Department of Clinical Sciences
Lund University
LUND
SWEDEN
ABSTRACT

Hyaluronan (HA) is an abundant polysaccharide found in the extracellular matrix; it is essential for the maintenance of normal tissues, but it also promotes cancer progression by creating a favorable microenvironment to allow the growth of tumor cells. HA is synthesized by specific plasma membrane-bound enzymes, hyaluronan synthases (HAS 1-3), producing a chain made up of repeating units of N-acetylglucosamine (GlcNAc) and glucuronic acid (GlcUA). Extracellular vesicles (EVs) (including microvesicles, exosomes and apoptotic bodies) are membrane-derived extracellular particles that contain and transfer cytosolic components, proteins, RNA, ribosomes and selected plasma membrane proteins. The EVs that originate from cancer cells carry characteristics of their cellular origin and may serve as a surrogate for tumor biopsies, enabling real-time diagnosis and disease monitoring. It is surprising that even though all the body fluids known to contain an abundance of HA, such as synovial fluid, plasma and ascites of cancer patients, are rich in EVs, the data included in this thesis is the first time that EVs have been demonstrated to act as special carriers of HA.

This thesis aimed at achieving a more profound understanding of the functional relevance of HA synthesis and its effect on the secretion of EVs. Initially we observed that bone marrow derived mesenchymal stem cells secreted high levels of endogenous HA, part of which was associated with the EVs. These HA coated MSC-EVs may be one of the factors mediating tissue regeneration and wound healing e.g. the interaction of these particles with other cells could be one mode of intercellular communication. Overexpression of GFP-HAS3 in MV3 metastatic melanoma cells promoted high levels of HA and EV secretion. Furthermore, enhancing the levels of UDP sugars not only increased the duration and proportion of GFP-HAS3 residing on the plasma membrane but it was also associated with released EVs, termed as HAS3-EVs. When the levels of UDP-sugars levels declined, an opposite effect was observed in the EV secretion and HAS3 plasma membrane residence duration and proportion. Our results indicated that the presence of HAS3 on the plasma membrane was required for HA secretion and its release in the EVs.
The HAS3-EVs released by MV3 metastatic melanoma cells were able to induce tumorigenic properties in their target cells, HaCaT (keratinocytes) and WM115 (melanoma). We observed that HAS3-EVs carry IHH (Indian Hedgehog) ligands which interacted with the target cells and were able to induce the hedgehog signaling (HH) pathway. The downstream target of the HH pathway, c-Myc, was upregulated with the subsequent expression of claspin. This signaling axis evoked an increase in proliferation, invasion and epithelial to mesenchymal transition in the target cells. In vivo staining of melanoma tissue sections revealed a correlation in the expression pattern between HA and claspin. The presence of IHH ligands in the EVs was associated with the HA synthesis rate of the donor melanoma cells. Inhibition of HH signaling also affected HA synthesis in melanoma cells. Inhibition of HA synthesis or HH signaling in MV3 melanoma cells resulted in decreased incorporation of IHH in HAS3-EVs. Conversely, increased HA synthesis increased the association of IHH with HAS3-EVs. The differential levels of IHH in HAS3-EVs were directly proportional to the level of HA synthesis and the proliferation rate in the target cells. The positive feedback mechanism displayed by HA and HH pathways in melanoma is a novel finding emerging from this study. In summary, the results presented in this thesis reveal details of the molecular mechanisms involved in HAS3 trafficking and the related HA synthesis and its association with EV production. Moreover, we unraveled the signaling axis activated by IHH which was mediated by HAS3-EVs in the target cells and we also observed that HAS3-EVs were capable of inducing tumorigenic properties in target cells. The observations that MSCs and tumor cells secrete HA coated EVs and the fact that the components carried by these particles have the ability to change the nature of target cells are significant findings. These results suggest that HA coated EVs could be utilized as therapeutic and non-invasive prognostic tools.
Hyaluronaani (HA) on soluväliaineen yleisin polysakkaridi, joka ylläpitää normaalien kudosten tasapainoa, mutta myös edistää syövän etenemistä luomalla suotuisan mikroympäristön syöpäsolujen kasvulle. HA-ketju rakentuu toistuvista ala-yksiköistä, N-asetyylilukosamiini (GlcNAc) ja glukuronihaposta (GlcUA) ja sitä tuottavat erityiset solukalvoilla sijaitsevat entsyymit, hyaluronaanisyntaasit (HAS 1-3). Solunulkoiset vesikkelit (EV:t) (mikrovesikkelit, eksosomit ja apoptoosikappaleet) ovat solukalvoista kuroutuvia solunulkoisia rakkuloita, jotka kuljettavat ja siirtävät soluliman molekyylejä, kuten RNA:ta ja solukalvoproteiineja solujen välillä. Syöpäsolulista peräisin olevat EV:t kantavat samoja molekyylejä kuin alkuperäinen solu, joten ne mahdollistavat reaaliaikaisen sairauksien seurannan.

On kiehtovaa, että kaikki HA-pitoiset kehon nesteet, kuten nivelneste, plasma ja syöpäpotilaiden askiittineste, sisältävät paljon EV:iä, mutta kukaan ei ole ennen osoittanut, että ne voisivat toimia erityisinä HA:n kantajina.


Luokitus: QU 83, QU 350, QU 375, QZ 203, QZ 360
Yleinen suomalainen ontologia: hyaluronaani; kantasolut; geeniekspressio; syöpäsolut; melanooma; etäpesäkkeet; solukalvot; soluviestintä
(Flourishment of a lotus plant depends on its water level. Likewise gaining knowledge depends on one’s motivation)

- Thiruvalluvar (Thriuvalluvar)
  அகாலத்தெரு: 60, குறள்: 595 (Chapter: 60, Couplet: 595) | Dated 300 BC
ACKNOWLEDGEMENTS

This thesis work was carried out in the Institute of Biomedicine/Anatomy, School of Medicine, at the University of Eastern Finland. It has been a great challenge for me at both the personal and academic levels to complete this thesis. I owe my gratitude to all the amazing people who have helped me throughout these years and influenced my life and this work.

I would like to express my sincere gratitude to my supervisor Docent Kirsi Rilla for her immense kindness, support, motivation and patience throughout my PhD studies. I would like to thank you for providing me with such a wonderful scientific environment and nurturing me to be an independent scientist. While I struggled in the initial years to find a foothold in this field, you gave me the ray of hope that doing a PhD could be a pleasant journey. I am deeply thankful to you for that and I cannot have imagined having a better thesis supervisor than you. I would also like to thank my second supervisor, Docent Sanna Oikari. I am indebted for your constructive comments and suggestions on my thesis. You have always been willing to share your knowledge with me and it has helped me in many ways.

My most heartfelt thanks to Professor Emeritus Markku Tammi and Professor Emerita Raija Tammi for the encouragement, support and introducing me to the field of hyaluronan biology and salibandy. You gave me the backing I required as a young researcher which helped me develop into the researcher I am now. Hope in the future I will be half as good as you are in both doing research and playing salibandy. Thank you for your encouragement towards my work.

I sincerely thank my thesis reviewers, Professor Johanna Ivaska and Professor Aki Manninen, for your valuable comments and extensive reviews. I am also grateful to Dr. Ewen MacDonald for his careful and valuable revision of the language in this thesis.

I would like to extend my gratitude to the personnel of the Biomedicine Laboratory, Riikka Kärnä, Eija Vartianiemi Eija Rahunen, Karoliina Tenkanen and Kari Kotikumpu. Riikka, you have been a wonderful person, always ready to help and I have always been amazed at the speed in which you execute these experiments. I thank you all for helping me and not saying no to any of my requests. I would like to acknowledge Arja Afflekt for helping me with the paperwork surrounding this thesis. Warm thanks to my co-authors Dr. Ashik Jawahar Deen, Dr. Piia Takabe, Dr. Kai Härkönen, Dr. Raquel Melero, Dr. Sanna Pasonen-Seppänen, Dr. Elisa Lazaro Ibanez, Dr. Pia Siljander, Sara Wojciechowski, Johanna Matilainen M.Sc, Riikka Kärnä M.Sc and Dr. Arto Koistinen.
One of the best things that happened to me during these years is the privilege to enjoy the friendship and support of so many people. I would like to take this opportunity and thank all of them. Raquel, you were my first friend here in Kuopio and thanks for all the support you gave in my early days. It helped me to survive the tough times. I want to thank Piia Takabe for always motivating me with her cheerful face even in tough times and for sharing thoughts on troubleshooting experiments. Kai Härkönen, it has been a great pleasure to share the office space and research interest in EVs with you. Thank you for all the fun-filled trips and long talks that we enjoyed. Johanna Matilainen, I wish we could have had more time working together but I am happy to have your amazing friendship. Thank you for being immensely supportive and you have cheered me up on many of my bad days with your encouraging words. I would like to thank Tommi Paakkonen for all the enjoyable chats and for taking care of Kai. I would also like to thank Sanna Pasonen-Seppänen for her valuable comments on my work and encouraging me at all times. I have had the joy of working with Leena Rauhala and Lasse Hämäläinen and I would like to thank them for sharing their research ideas with me. I would like to extend my gratitude to Silja Pyysalo, Virpi Tiitu, Kirsi Kainulainen, Taija Hukkanen, Heikki Kyykallio, Janne Capra, Sanjeev Ranjan and Kari Törrönen for their friendship.

I express my deepest gratitude to my current supervisor Dr. Minna Kaikkonen-Määtä for including me in her group and giving me the opportunity to explore the field of cardiovascular genomics. You and everyone in the group have made me feel comfortable and helped me find a balance between my current lab work and this thesis preparation. I am very thankful to my CAD group members Pierre, Anu, Tiit, Aarthy, Kadri, Mykael, Vaneesa, Nick, Ilakya, Oscar, Tuula and Abhishek for sharing the funfilled moments.

I would like to thank my Indian friends here in Kuopio. Rolls, Ashok, Rajasekar, Shalem, Merlin, Bhavik, Bhavin, Rammohan, Raghu, Yashu, Vijalakashmi, Jagadish, Sireesha, Arun, Prashanti, Varsha and Narasinha Shurpali for making my social life a delight. Special thanks to Krish and our lovable naughty buchiki Reyna for the unconditional friendship. You both added a different color of happiness to my life which has boosted my spirits in all aspects. My heartfelt gratitude to my Swedish family Åsa, Adam and Staffan for the warm nurture and care you provided during my stay in Uppsala. The times we shared shall always remain immortal in my memory and thanks for sharing the happiness with me. I want to thank my friends from Uppsala Praveen, Jay, Mals, Kalpana, Keerthana, Sharan, Vidya, Kalai and Divya. You guys gave me one of my happiest times. My heartfelt thanks to Divya for making me feel at home and showing unlimited affection. I would like to thank my Madha college friends, Saranya, Dhivya, Joyce, Aakash, Thinesh and Naveen for your priceless friendship of 14 years. Naveen, thank you for the emotional support over the years.
Ashik, I cannot thank you enough. The dedication and love you have towards research has inspired me constantly. Your optimistic attitude and unconditional support made me go through this PhD with ease. You have been with me through all the ups and downs both professionally and personally. This life we have together is a boon and I couldn’t possibly ask for more. Thank you for your immense kindness, patience, care and making me extremely happy.

An acknowledgement would not be complete without thanking my family. My heartfelt thanks to my dad Thanigai Arasu for his never-ending support and love. Special thanks to my mom Kanchana, the iron lady behind my success. You taught me to be strong and never give up, which is what made me what I am today. You and dad went through a lot of hardships to give me and Thyagu a happy, nurturing family a kid requires and we will always be grateful to you for that. Thyagu, as my little brother, your affection for me helped me strive through these years away from home. Thank you for your unconditional love and always keeping the childish happiness in us alive. I would also like to thank my grandparents here. I feel sad when I think that you are not here to see all that has happened in my life. But I am sure that you are watching over me and proud of my achievements. I am blessed to have a family like this and thank you all for your love and support.

The Academy of Finland, Jane and Aatos Erkko Foundation, Centre for International Mobility (CIMO), Matti and Vappu Maukonen Foundation, K. Albin Johanssons stiftelse Foundation, Paavo Koistinen Foundation, Kuopio University Foundation, Northern Savo Cancer Foundation and Otto A. Malm Foundation supported this work financially.

Time and place/
signature/
LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications:


The publications were adapted with the permission of the copyright owners.
ADDITIONAL PUBLICATIONS

List of additional publications not included in the thesis:

I. Koistinen V, Härkönen K, Kärnä R, Arasu UT and Rilla K. EMT induced by
EGF and wounding activates hyaluronan synthesis machinery and EV

II. Lazaro-Ibanez E, Neuvonen M, Takatalo M, Arasu UT, Capasso C, Cerullo
V, Rhim JS, Rilla K and Yliperttula M, Siljander PR. Metastatic state of
parent cells influences the uptake and functionality of prostate cancer cell-

III. Rilla K, Mustonen AM, Arasu UT, Härkönen K, Matilainen J and Nieminen
P. Extracellular vesicles are integral and functional components of the

IV. Melero-Fernandez de Mera M, Arasu UT, Kärnä R, Oikari S, Rilla K, Vigetti
D, Passi A, Heldin P, Tammi MI and Deen AJ. Effects of mutations in the
post-translational modifications sites on the trafficking of hyaluronan

V. Arasu UT*, Härkönen K*, Koistinen A and Rilla K. Correlative light and
electron microscopy is a powerful tool to study interactions of extracellular

VI. Härkönen K, Oikari S, Kyykallio H, Capra J, Hakkola S, Ketola K, Arasu
JM and Rilla K. CD44s assembles hyaluronan coat on filopodia and
extracellular vesicles and induces tumorigenicity of MKN74 gastric
# CONTENTS

ABSTRACT ........................................................................................................... 7
TIIVISTELMÄ....................................................................................................... 9

ACKNOWLEDGEMENTS ...................................................................................... 13
CONTENTS ............................................................................................................ 19

1 INTRODUCTION ............................................................................................... 23

2 REVIEW OF LITERATURE .................................................................................. 27
  2.1 HYALURONAN ............................................................................................. 27
      2.1.1 Hyaluronan - Discovery, structure and properties .............................. 27
      2.1.2 Hyaluronan synthases - Discovery, structure and properties .......... 27
      2.1.3 Biosynthesis and regulation of hyaluronan synthesis ...................... 29
      2.1.4 Biosynthesis and regulation of UDP sugar pools ............................ 34
      2.1.6 Biological functions of hyaluronan .................................................. 37
  2.2 HYALURONAN AND CANCER ..................................................................... 40
      2.2.1 Melanoma .......................................................................................... 42
      2.2.2 Mesenchymal stem cells ................................................................... 43
  2.3 EXTRACELLULAR VESICLES ..................................................................... 44
      2.3.1 Biogenesis and secretion of EVs ...................................................... 45
      2.3.2 Molecular composition of EVs ......................................................... 47
      2.3.3 EVs role in cell to cell communication ........................................... 49
  2.4 EVs IN CANCER .......................................................................................... 50
      2.4.1 Cancer diagnosis and prognosis using EVs ...................................... 55
  2.5 HEDGEHOG SIGNALING PATHWAY AND CANCER ................................. 56

3 AIMS OF THE STUDY ....................................................................................... 59

4.1 MATERIALS .................................................................................................... 61
  4.1.1 Cell lines ............................................................................................... 61
  4.1.2 Human tissue samples ......................................................................... 61

4.2 METHODS ....................................................................................................... 62

5 RESULTS ........................................................................................................... 65

5.1 INFLUENCE OF HAS3 TRAFFICKING ON EV SECRETION IN MV3
  MELANOMA CELLS OVEREXPRESSING GFP-HAS3 ..................................... 65
      5.1.1. UDP-sugars on HAS3 trafficking ................................................... 65
      5.1.2. HAS3 trafficking and plasma membrane residence controls release of
             HAS3-EVs ......................................................................................... 66
  5.2 HA COATED EVs FROM MSCs ................................................................. 67
      5.2.1 Composition and structure of EVs released from hMSCs ............... 67
      5.2.2 Effect of HAS enzymes on hMSCs and its EVs ............................... 68
5.3 MELANOMA CELLS DERIVED HAS3-EVs AND THEIR FUNCTIONS .... 69
  5.3.1. GFP-HAS3 EVs: Characterization and functions exerted on target
cells .................................................................................................................. 69
  5.3.2. Comprehensive profiling of HAS3-EVs treated recipient cells for
identification of key players ........................................................................ 70
5.4 HAS3-EVs REGULATION OF CELL PROLIFERATION DEPENDS ON IHH
MITOGEN MEDIATED MYC AND CLASPIN EXPRESSION ......................... 71
  5.4.1. HAS3-EVs stimulate HH signaling and thereby upregulation of c-Myc
and claspin ......................................................................................................... 71
  5.4.2. HAS3-EVs carry IHH to stimulate the HH signaling pathway .......... 71
5.5. HA AND HH PATHWAYS HAVE A POSITIVE FEEDBACK REGULATION
...................................................................................................................... 72

6 DISCUSSION AND CONCLUSION .............................................................. 73
  6.1 EVs GOVERNED BY MESENCHYMAL STEM CELLS ......................... 73
  6.2 EFFECT OF HAS3 RECYCLING IN THE SECRETION OF EVs .......... 75
  6.3 HAS3-EVs TRIGGER A SIGNALING PATHWAY IN RECIPIENT CELLS 76
  6.4 A NOVEL FEEDBACK REGULATION BETWEEN HH AND HA ........... 78
  6.5 HA CARRYING EVs AS BIOMARKERS .............................................. 79
  6.6 CONCLUSIONS AND FUTURE DIRECTIONS ................................. 82

REFERENCES .................................................................................................. 85

ORIGINAL PUBLICATIONS (I – III) ................................................................. 117
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alix</td>
<td>ALG-2-interacting protein</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>CLEM</td>
<td>Correlative light and electron microscopy</td>
</tr>
<tr>
<td>CD44</td>
<td>Cluster of differentiation 44/hyaluronan receptor</td>
</tr>
<tr>
<td>DHH</td>
<td>Desert hedgehog</td>
</tr>
<tr>
<td>EV</td>
<td>Extracellular vesicle</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ELSA</td>
<td>Enzyme linked sorbent assay</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic stem cells</td>
</tr>
<tr>
<td>ESCRT</td>
<td>Endosomal sorting complex required for transport</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GFAT</td>
<td>Glutamine fructose-6-phosphate amidotransferase</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>GlcUA</td>
<td>Glucuronic acid</td>
</tr>
<tr>
<td>Gli</td>
<td>Glioma associated oncogene</td>
</tr>
<tr>
<td>GNPDA</td>
<td>Glucosamine-6-phosphate deaminase</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronan</td>
</tr>
<tr>
<td>HYAL</td>
<td>Hyaluronidase</td>
</tr>
<tr>
<td>HH</td>
<td>Hedgehog</td>
</tr>
<tr>
<td>HAS</td>
<td>Hyaluronan synthase</td>
</tr>
<tr>
<td>HABC</td>
<td>Hyaluronan binding complex</td>
</tr>
<tr>
<td>IHH</td>
<td>Indian hedgehog</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>NTA</td>
<td>Nanoparticle tracking analysis</td>
</tr>
<tr>
<td>O-GlcNAc</td>
<td>O-linked-N-acetylglucosamine</td>
</tr>
<tr>
<td>OGT</td>
<td>O-GlcNAc transferase</td>
</tr>
<tr>
<td>PTCH</td>
<td>Patched</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SMO</td>
<td>Smoothened</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short interfering RNA</td>
</tr>
<tr>
<td>SP1/3</td>
<td>Specificity protein 1/3</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
</tr>
<tr>
<td>4MU</td>
<td>4-methylumbelliferone</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

During development in multicellular organisms, the multitude of physiological and pathological processes demands efficient intercellular communication (Majka et al., 2001). Communication between the cells is mediated either via autocrine, paracrine or juxtacrine signaling. Recent studies indicate that the communication between cells and tissues can be mediated also by extracellular vesicles (Ratajczak et al., 2006; Camussi et al., 2010). Extracellular vesicles (EVs) are membrane enclosed sacs that carry functional cargo such as nucleic acids, proteins, lipids and probably several other cellular components (Raposo and Stoorvogel, 2013). EVs are found in biological fluids and are secreted by different cell types. The cargo carried by the EVs can be ferried between cells which means that the EVs have clinical implications such as functioning as noninvasive biomarkers for diagnosis and therapy (Van Niel et al., 2018). Additionally, the EVs released from mesenchymal stem cells have been shown to enhance wound healing, regeneration and tissue repair (Collino et al., 2010). On the other hand, EVs released from cancer cells have been demonstrated to participate in the acquisition of cancer hallmark properties, such as invasion, angiogenesis, tumor proliferation and metastasis (Kim et al., 2003; Janowska-Wieczorek et al., 2005; Al-Nedawi et al., 2008). Understanding both the mechanisms of the biogenesis of cancer EVs as well as their mode of action on healthy cells would help to understand the disease progression and hopefully contribute to the development of cancer therapy and treatment.

Hyaluronan (HA) is a ubiquitous, high molecular weight glycosaminoglycan consisting of alternating disaccharide units of N-acetyl-D-glucosamine (GlcNAC) and D-glucuronic acid (GlcUA) (Fraser, Laurent and Laurent, 1997). In vertebrates, HA is produced on the inner surface of the plasma membrane by a family of HA synthases (HAS1-3) that extrude the growing HA chain into the extracellular space. A single HA molecule can reach a molecular weight of up to 10 million Daltons with an extended chain length of 22.5 µm (Stern, 2009b). HA synthesis can be regulated either by transcriptional regulation of HAS genes or by post-translational modification of HAS proteins. The synthetic activity is also dependent on the cytosolic levels of precursor sugars UDP-GlcNAC and UDP-GlcUA. The cellular levels of UDP-sugars can be influenced by chemical compounds such as 4-MU (4-methylumbelliferone), mannose and glucosamine and thereby manipulating the activity of HA synthesis (Jokela et al., 2008; Tammi et al., 2011). Mannose and glucosamine affect the UDP-GlcNAc pool by shuttling the glycoconjugate in the
hexosamine biosynthesis pathway by directly acting as a substrate. 4-MU depletes UDP-GlcUA pool by forming a 4-MU glucuronide conjugate (Vigetti et al., 2009a; Tammi et al., 2011). These chemical compounds can affect enzymatic activity of HAS2 and O-GlcNAc transferase (OGT), and also regulate transcription of HAS2 and HAS3 – thereby representing another route of regulation of HA synthesis (Kultti et al., 2009; Jokela et al., 2011; Vigetti et al., 2012a). HA can be pericellularly bound to the HASs during synthesis or to its plasma membrane receptors HARE, CD44, LYVE-1 and ICAM-1. The anionic nature and negative charge of HA enables it to bind large quantities of water and this aids in acting as a space filler in tissues (Zhou et al., 2003; Toole, 2004; Jiang, Liang and Noble, 2011; DeAngelis, 2012). The unique physiochemical properties of HA allow it to form a pliable matrix during embryonic development to promote tissue remodeling (Toole, 2001). This property of HA is required in certain circumstances e.g. during atrioventricular canal morphogenesis where endothelial cells migrate and transform into mesenchymal cells (Camenisch et al., 2000a).

Due to its growth promoting properties, HA plays an important role both in embryonic development and cancer progression (Stern, 2009a). HA is associated with the aggressive nature of numerous cancers such as ovarian, pancreatic and breast (Knudson, 1993). During malignant transformation, there is a transcriptional switch in the HAS isoforms accompanied by alterations in HA production, leading to changes in the extracellular environment and further on to deranged cell-to-cell interactions and oncogenic transformation. The role of HA in cancer is controversial as elevated levels of HA correlate with poor prognosis in colorectal, breast, prostate, gastric and ovarian cancers (Ropponen et al., 1998; Auvinen et al., 2000; Posey et al., 2003; Sironen et al., 2011), whereas decreased levels of HA correlate with poor prognosis and tumor grade in squamous cell carcinoma of the skin, larynx, lung and mouth (Tammi et al., 2008). This shows that the HA content of tumors may exhibit either a positive or a negative correlation with poor prognosis and tumor grade.

It has been shown recently that the overexpression of hyaluronan synthase 2-3 causes the formation of numerous slender plasma membrane protrusions and induces shedding of EVs covered with a thick layer of HA (Kultti et al., 2006a; Rilla et al., 2017) This thesis work aimed to investigate the factors influencing the production of HA-coated EVs and to elucidate their mechanism of action in recipient cells. The residence time of GFP-HAS3 in the plasma membrane and its influence on EV secretion were studied by manipulating the intracellular traffic of
GFP-HAS3 in MV3 melanoma cells with inducible GFP-HAS3 expression. Substrates of HAS3 i.e. UDP-GlcNAc and UDP-GlcUA and O-GlcNAc post-translational modification of GFP-HAS3 could positively regulate its plasma membrane presence and secretion in EVs. MV3 cell line with an inducible expression of GFP-HAS3 produced increased levels of EVs carrying GFP-HAS3 and HA coat; these EVs were labelled as HAS3-EVs. Secretion of these HAS3-EVs was decreased with depletion of the cellular levels of HAS3 substrates i.e. UDP-GlcUA and UDP-GlcNAc. The results thus point to a role of HAS activity as an inducer of EV shedding. HAS3-EVs were found to carry CD44, GFP-HAS3, IHH, DHH and EGF and they were able to trigger the hedgehog signaling pathway in normal keratinocyte cells (HaCaT). When EVs interact with HaCaT and WM115 (melanoma) cells, claspin was identified as a potential target downstream of c-Myc, aiding the cells to undergo increased proliferation, which is a hallmark of cancer. In addition, EVs secreted by human mesenchymal stem cells carry a high content of HA on their surface. This analysis delineated the fact that stem cell-derived EVs carrying HA could be involved in paracrine signaling of the extracellular matrix (ECM) remodeling. The results presented in this thesis are intended to widen our knowledge of the EVs secreted from cells with high amounts of HA, which could be a underlying signaling mechanism exerting tissue regeneration in normal cells while being hijacked by cancer cells to promote uncontrolled proliferation.
2 REVIEW OF LITERATURE

2.1 HYALURONAN

2.1.1 Hyaluronan - Discovery, structure and properties

The history of hyaluronan began in 1841 when Henle named the amorphous material between cells as “ground substance” (Henle 1841), which was later renamed as “acid mucopolysaccharides” by Karl Meyer. In 1934, Meyer and Palmer subsequently identified the hexosamine containing sugar polymers as hyaluronan (HA) which was discovered in the vitreous body of the bovine eye both unbound as well as bound to proteins (Meyer K and Palmer J, 1934). HA, a linear sugar polymer and ubiquitous glycosaminoglycan (GAG) consists of repeating disaccharide units of GlcNAc (N-acetyl-D-glucosamine) and GlcUA (D-glucuronic acid) forming $[\beta1,3-N$-acetyl-D-glucosamine-β1,4-D-glucuronic acid-]_n$ (Toole, 2004). HA chains are synthesized in the cellular plasma membrane and the chains have a length of 2-25 µm consisting of up to 25,000 disaccharide units corresponding to a relative molecular mass of $10^6$ - $10^7$ Da. HA is a unique molecule because it is synthesized on the plasma membrane unlike other GAGs that are synthesized in the Golgi apparatus. Additionally, HA is non-sulfated and it is not covalently linked to a core protein, but it can organize the pericellular and extracellular matrix by binding to proteoglycans and other proteins (Fraser et al., 1997). HA has one carboxyl group per repeating disaccharide unit, making it hydrophilic and a polyelectrolyte with a negative charge at neutral pH (Scott, 1989). Due to these unique physiochemical properties, HA binds water molecules and forms viscous gels at relatively low concentrations, creating a pliable matrix in the extracellular environment and thereby making it important for tissue homeostasis and biomechanical integrity (Fraser, Laurent and Laurent, 1997).

2.1.2 Hyaluronan synthases - Discovery, structure and properties

In 1993, the first hyaluronan synthase (HAS) gene, *HasA*, was discovered and cloned in *Streptococcus pyogenes* (DeAngelis, Papaconstantinou and Weigel, 1993). In mammals, the HAS gene family is highly conserved with three different hyaluronan synthases HAS1 (Shyjan et al., 1996), HAS2 (Watanabe and Yamaguchi, 1996) and HAS3 (Spicer and McDonald, 1998) located in chromosomes 19 (q13.3-13.4), 8 (q24.2) and 6 (q22.1), respectively. The human HASs share 55–71% sequence identity and nearly 25% amino acid identity with *Streptococcus pyogenes HasA* and
they all catalyze the synthesis of HA (Spicer and McDonald, 1998; Tammi et al., 2011). Hyaluronan synthases (HAS) are multispans transmembrane enzymes whose central domain consists of the catalytic unit. The mammalian HASs consist of 7 membrane domains that include 6 transmembrane domains and 1-2 membrane associated domains (Weigel et al., 1997). While the 3D protein structure of the human HASs remains to be resolved, in silico and in vitro methods have deduced the genomic structure, revealing that HAS1 has 5 exons with 2 slightly different transcripts, HAS2 has 4 exons and HAS3 has 8 exons, of which only 4 exons are coding sequences for transcription. HAS3 has 3 distinct transcripts, and of those, two transcripts, HAS3v1 and HAS3v2 show similarities to HAS2. The only difference between HAS3v1 and HAS3v2 is that the last coding exon i.e. exon 4 in HAS3v2, is shorter, resulting in a C-terminally truncated and different protein than HAS3v1 (Monslow et al., 2003). This shared pattern among the HASs suggests that their genes must have evolved from a common ancestral gene. The activation and localization of the HAS genes may also be influenced by alternative splicing. HAS1 variants have been detected in multiple myeloma (Adamia et al., 2005), bladder cancer (Golshani et al., 2007) and Waldenström’s macroglobulinemia (Adamia et al., 2003).

It has been observed in Xenopus that during embryonic development, Has1 and Has2 expression is homogenously spread throughout the embryo, while Has3 is localized in the cement gland and inner ear (Camenisch et al., 2000a; Tammi et al., 2011). During mouse embryonic development, Has1 disappears on day 8.5, Has2 expression is seen in all stages while Has3 expression is observed only in the later stages of embryonic development. In fact, the functional requirement of each Has in mice development varies. Although HAS2 is considered as an important isoform in many cell types (Jacobson et al., 2000), recent studies indicate that HAS1 is required in inflammation (Stuhlmeier and Pollaschek, 2004; Chang et al., 2014; Siiskonen et al., 2014), while HAS3 is associated with the development of cardiomyopathy (Teng et al., 2011), cancer progression (Chang et al., 2015) and normal brain function (Arranz et al., 2014). HAS1 shows very low affinity towards UDP-sugar substrates whereas HAS3 exhibits the highest affinity (Tammi et al., 2011; Rilla et al., 2013). As cytoplasmic concentrations of these UDP-sugars can vary, this differential affinity can mean that the HAS isoforms do not necessarily have the same enzymatic activity. Even though the cellular concentration of UDP-GlcNAc is 2-17 times higher than that of UDP-GlcUA, the affinity of HAS enzymes towards UDP-GlcUA is 3-14 times more than with UDP-GlcNAc (Pummill and DeAngelis, 2002). The molecular weight of synthesized HA chains depends on the HAS isoform involved
in their production. As shown in COS1 cells transfected with HAS isoforms, HAS1 and 2 produced HA with molecular weights of 2x10^5-2x10^6 Da while HAS3 produced HA with lower molecular weights (1x10^5-1x10^6 Da) (Itano et al., 1999). In addition, in aortic smooth muscle cells HAS1 and HAS2 produced higher molecular weight HA (2-10x10^6 Da), and HAS3 synthesized lower molecular weight HA (2x10^6 Da) (Wilkinson et al., 2006). However, a different result was obtained with CHO cells, where HAS2 produced a higher molecular weight HA (3.9x10^6 Da) in comparison to HAS1 and HAS3 (0.12x10^6-1.0x10^6 Da) (Brinck and Heldin, 1999; Itano et al., 1999). HAS’s ability to synthesize HA chains can be influenced by post-translational modifications, the cellular environment and intracellular trafficking of HASs. The reason behind the differential ability of HAS to synthesize HA with different molecular weight is still unclear.

2.1.3 Biosynthesis and regulation of hyaluronan synthesis

Biosynthesis of hyaluronan
In 1959, Markovitz et al., first observed HAS activity in cell homogenates (Markovitz, 1959), but it was not until 1984 that it was discovered that in contrast to the other GAGs, HA synthesis occurs in the inner face of the plasma membrane (Prehm, 1984). The HAS enzyme travels to the plasma membrane, where it exerts its catalytic activity, from the endoplasmic reticulum via the Golgi apparatus (Rilla et al., 2005). HASs do not carry the usual N-terminal signal which means that they are not processed via the conventional secretory pathway through the Golgi complex (Rilla et al., 2005). The residence of HASs on the plasma membrane is crucial and they utilize UDP-N-acetylglucosamine and UDP-glucuronic acid as substrates along with Mg^{2+} or Mn^{2+} to synthesize the HA chains (Weigel and Deangelis, 2007). While HASs do not require primers to initiate HA polymerization, at the start, there is a kinetic lag denoting that initiation of HA synthesis is the rate limiting phase (Baggenstoss and Weigel, 2006). Even though it has been shown that synthesis is initiated at the plasma membrane (Rilla et al., 2005), there are also indications of HAS activity in the intracellular compartment during in vitro analysis of the membrane fractions (Vigetti et al., 2009). In Xenopus laevis, HAS utilizes the non-reducing end to add precursors (Bodevin-Athelet et al., 2005), while in humans and mice, the precursor sugars are added to the reducing end of the growing HA chain (Prehm, 1983a, 1983b). A single HAS protein can produce only one HA molecule as the lifetime of HAS protein is 4-5 hours (Kitchen and Cysyk, 1995). Based on this value, it was estimated that the rate of HA synthesis would be 3 monosaccharides per second which in approximately 3 hours would yield a 6x10^6
Da HA chain (Karvinen et al., 2003; Pummill and DeAngelis, 2003). Though the HA chain may remain attached to the HAS during synthesis, multiple interactions like fluid currents, Brownian motion and matrix proteins in the extracellular environment may remove HA off the enzyme (Weigel and Baggenstoss, 2012).

Figure 1: Biosynthesis and regulation of HA and HAS. As shown in the above figure UDP-GlcUA is inhibited using 4MU treatment while UDP-GlcNAc content is enhanced using glucosamine and inhibited using mannose treatment. O-GlcNAc modified SP and YY1 transcription factors bind to HAS2 promoter region. O-GlcNAc modified HASs are transported from Golgi to the plasma membrane. Once in the plasma membrane HASs form dimers and possibly multimers by ubiquitination modification and initiate HA synthesis. If they are not required in the plasma membrane they are transported into endosomes by Rab10 GTPase (Deen et al., 2014). Hyaluronan synthesis takes place in the plasma membrane and the growing chain is extruded into the pericellular and extracellular space.
Although in fibroblasts it was proposed that HA would be exported with the help of ABC transporters like MRP5 (Schulz et al., 2007), in human breast cancer cells, it was found that the ABC transporters did not contribute to HA translocation (Thomas and Brown, 2010). With the energy produced from glycosyltransferase activity, two or more HAS molecules can form dimers or multimers and a transmembrane pore, from which HA is extruded (Karousou et al., 2010a; Hubbard et al., 2012; Bart et al., 2015).

**Regulation of hyaluronan synthesis**

The regulation of HA synthesis is very important due to the involvement of HA in numerous physiological and pathological events such as inflammation, cell migration, embryogenesis, cell migration and cancer. HA synthesis can be influenced by the presence of several endogenous factors as well as by synthetic compounds. Its regulation can be considered to occur in various stages: 1) transcriptional regulation of HAS 2) post-translational modification of HAS activity and 3) altering the availability of HA precursor sugars.

**Transcriptional regulation of HAS**

Various studies have detected a correlation between HA synthesis and HAS mRNA levels, indicating that the expression of HAS enzymes is an important determinant of the HA synthesis rate (Pienimäki et al., 2001; Karvinen et al., 2003). The transcriptional rate of HAS mRNA and hence HA synthesis activity can be altered by several factors like cytokines, hormones, growth factors and synthetic compounds (Jacobson et al., 2000; Karvinen et al., 2003; Yamada et al., 2004). The response of HAS isoforms to these external stimuli is dependent on many factors such as cell type, development stage and treatment conditions (examples below) (Jacobson et al., 2000). One of the most powerful regulators of keratinocytes is epidermal growth factor (EGF), which exerts a stimulatory effect on HA synthesis and cell behavior via its receptor (EGFR) (Piepkorn et al., 1998). HAS2 is the primary HAS gene responding to EGF stimuli (Saavalainen et al., 2005) and together with keratinocyte growth factor (KGF) in monolayer and organotypic cultures, the mRNA levels of both HAS2 and HAS3 are increased (Sayo et al., 2002; Karvinen et al., 2003; Pasonen-Seppänen et al., 2003). However, the differential response of HASs to transforming growth factor (TGF-β) is interesting, as in keratinocytes, the HAS1 level was increased while HAS2 and HAS3 levels declined (Sugiyama et al., 1998; Pasonen-Seppänen et al., 2003). In vascular endothelial cells, TGF-β treatment increased HAS2 mRNA and protein levels, and in fibroblasts both HAS1 and HAS2 mRNA levels increased due to TGF-β treatment (Suzuki et al., 2003; Stuhlmeier and
Pollaschek, 2004). The transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), mediates the effects of cytokines like interleukin (IL)-1β which act as inducers of HAS1 mRNA expression in synoviocytes (Stuhlmeier and Pollaschek, 2005; Kao, 2006). In dermal fibroblasts and osteoblasts, the mRNA expression and stability of HAS2 are downregulated by hormones such as hydrocortisone and other glucocorticoids (Jacobson et al., 2000; Zhang et al., 2000). Furthermore, in dermal fibroblasts, cytokines like IFN-γ, TNF-α and IL-1β induce the transcription of HASs (1-3) (Campo et al., 2006), in human periodontal ligament cells HAS2 and HAS3 transcription is induced by IL-1β and TNF-α (Ijuin et al., 2001) and in rabbit synovial membrane cells, the transcription of HAS2 and HAS3 mRNA is rapidly upregulated by cytokines IFN-γ, TNF-α and IL-1β (Tanimoto et al., 2001). HAS3 mRNA expression is increased in keratinocyte cells treated with IL-13, IL-14 and IFN-γ (Sayo et al., 2002; Ohtani et al., 2009).

EGF, retinoic acid (RA), platelet derived growth factor-BB (PDGF-BB), transcription factors like signal transducer and activator of transcription 3 (STAT3), cyclic adenosine monophosphate (cAMP) response element binding protein 1 (CREB1), SP (specificity protein) 1 and 3, ZBP1, E2FF, CREB, MZF1, NFκB, E-BOX and EGFR have binding sites in the proximal promoter regions of the HAS genes (1-3) and influence the HAS regulation (Jacobson et al., 2000; Monslow et al., 2003, 2004; Saavalainen et al., 2005; Makkonen et al., 2009). The significance of their regulation and differential splicing on both physiological and pathological conditions is being investigated. SMAD3 and SP3 have binding sites in the HAS1 promoter where TGF-β1 utilizes the former while IL-1β uses the latter during transcription (Chen et al., 2012). Has1 knockout mice do not display any structural and functional abnormalities and are also viable (Spicer and Nguyen, 1999; Kobayashi et al., 2010).

It has been reported that NFκB, NF-Y/CCAAT, SP1, SP3, STAT and retinoic acid receptor (RAR) are needed for transcriptional control of HAS2. In many mammalian tissues and cell types, HAS2 is the most abundant isoform (Tien and Spicer, 2005; Törrönen et al., 2014); its importance is indicated by the fact that Has2 knockout mice suffer severe defects in embryonic cardiac development and are not viable (Camenisch et al., 2000b). In human cells, HAS2 expression seems to depend on a short promoter sequence which is controlled by SP1 to achieve constitutive expression (Monslow et al., 2006). In addition to the above mentioned factors, it has been noted that a natural RNA interfering anti-sense HAS2 (AS-HAS2) enhances HAS2 mRNA expression in aortic smooth muscle cells and suppresses its expression in osteosarcoma cells (Michael et al., 2011; Chang et al., 2015). Furthermore, HAS2-AS1 can participate in chromatin remodeling around HAS2
promoter to initiate its open conformation (Vigetti et al., 2014). Under pathological conditions such as in cancer, HAS3 is very responsive to various stimuli (Tammi et al., 2005; Kultti et al., 2014a). Has3 knockout mice are viable even though the neurons are tightly packed because of the reduced HA content in hippocampus and the extracellular space which also makes these mice prone to epileptic seizures (Arranz et al., 2014). Transcription factors like SP1, NFκB and C/EBP are essential to initiate promoter activity of HAS3. Promoter activity of HAS3 was notably decreased when the binding site of Sp1 was disrupted, indicating that Sp1 appears to be an important regulator of HAS3 expression (Wang et al., 2015).

**Post-translational modification**

Regulation of HA synthesis by HASs is a complex process as it involves localization of the HAS in the plasma membrane where it has enzymatic activity and post-translational modifications like O-GlcNAcylation, ubiquitination, phosphorylation and N-glycosylation that influence the turnover time and activation of the HAS proteins (Vigetti et al., 2012b). O-GlcNAcylation carried out by O-GlcNAc transferase (OGT) occurs due to catalytic reaction creating β-O-linkage between N-acetylglucosamine (GlcNAc) and serine/threonine residue of proteins (Hart et al., 2007). Ser221 residue in the large intracellular loop of the HAS2 is O-GlcNAcylated. This suppresses the proteasomal degradation and leads to an increased half-life and more prolonged enzyme activity (Vigetti et al., 2012a; Melero-Fernandez de Mera et al., 2019). The O-GlcNAc modification of HAS3 increases the stability, activity, plasma membrane targeting and residence of this enzyme (Tammi et al., 2011). N-glycosylation can be inhibited by tunicamycin, which also increases HAS2 activity while evoking ER stress (Vigetti et al., 2009). In addition, HAS2 can be monoubiquitinated at the LYS190 residue, a modification required for the activity of HAS2. A point mutation of this residue renders HAS2 inactive (Karousou et al., 2010b). Phosphorylation of HAS can occur at multiple sites located in its cytoplasmic tail and intracellular domains (for example HAS3: Y347, Y333, T6 and Y329; HAS2: T412, Y326, S323, T110A) (www.phosphosite.org). Phosphorylation is thought to mainly elevate HAS enzymatic activity and HA synthesis, but the effect depends on the specific amino acid residue and isoform (Bourguignon et al., 2007; Vigetti et al., 2011). Phosphorylation of all HASs can occur via the heregulin (HRG/ErbB3/ErbB2) signaling pathway, leading to an increased HAS activity (Bourguignon et al., 2007). Conversely, HAS2 activity can be inhibited when AMP-activated protein kinase (AMPK) phosphorylates the THR110 residue (Bourguignon et al., 2007). In recent years, it has been shown that although the C-terminal domain is available for interactions, the N-terminal and
glycosyltransferase domains play a prominent role in HAS oligomerization leading to the formation of HAS homo- and hetero-dimers. The oligomerisation regulates the enzymatic activity of HASs and thus modulate the levels of HA synthesis. It has been postulated that the HAS1 homomeric complex has the lowest while that of HAS3 has the highest synthetic activity (Karousou et al., 2010b; Bart et al., 2015). This suggests that there are many alternative ways to regulate HAS activity and HA synthesis.

2.1.4 Biosynthesis and regulation of UDP sugar pools

**Biosynthesis**

The cell’s capacity to synthesize HA is reliant on the de novo production of HAS and on the availability of precursor sugars UDP-GlcUA and UDP-GlcNAc (Jacobson et al., 2000). As well as being used in HA synthesis, precursor sugars are consumed by other glycosaminoglycans (GAGs) that consist of glucuronic acids (GlcUA) and its isomer, iduronic acid (IdoA) and N-acetylgalactosamine (GalNAc) or N-acetylgalcosamine (GlcNAc) (Gandhi and Mancera, 2008; Afratis et al., 2012). Precursor sugars are synthesized in glucuronic acid and the hexosamine biosynthesis pathway respectively, with both pathways arising from glycolysis intermediates (Hanover, Krause and Love, 2012). After being added to the polymer, GlcUA in certain cases is isomerized into iduronic acid (IdoA) (Li, 2010). UDP-GlcUA synthesis starts with the conversion of the glycolysis intermediate, glucose-6-P to glucose-1-P by phosphoglucomutase. Glucose-1-P is then converted into UDP-glucose by UDP-glucose pyrophosphorylase (Fantus et al., 2006). The last, and rate limiting, step in this pathway is the formation of UDP-GlcUA, from UDP-glucose, which is catalyzed by UDP-glucose dehydrogenase (UDGDH). UDP-GlcNAc (uridine di phosphate N-acetylglucosamine) is synthesized in the hexosamine biosynthetic pathway (HBP). Its synthesis combines various cellular metabolic processes as it requires inputs from amino acids (glutamine), glucose derivatives (fructose-6-P), nucleotides (UTP) and fatty acids (acetyl-CoA) (Freeze and Elbein, 2009). HBP is related to various important cellular processes since its end product, UDP-GlcNAc, is utilized in the synthesis of proteoglycans, extracellular proteins with N- and O-linked oligosaccharides, glycosylphosphatidylinositol anchors (GPI), intracellular proteins with single N-acetylglucosamine (O-GlcNAcylation) and glycolipids. UDP-GlcNAc acts as the common precursor for both HA and O-GlcNAc post-translational modification of proteins (Fantus et al., 2006). The rate limiting step in this pathway is the formation of glucosamine-6-P from fructose-6-P, and any of the four enzymes, namely GFAT1-2 (glutamine fructose-6-phosphate
amido transferase 1 and 2) and GNPDA1-2 (glucosamine-6-phosphate deaminase 1 and 2) can catalyze this reaction. The last step in the synthesis is catalyzed by UDP-N-acetylglucosamine pyrophosphorylase, an enzyme adding UTP into precursor GlcNAc-1-P and forming UDP-GlcNAc. Apart from obtaining the sugar precursors from biosynthetic pathways, they can also originate from the degradation of glycoconjugate proteins in liver lysosomes and hence they can be reused by the cells (Varki et al., 2009).

**Regulation**

The cytosolic levels of the HA precursors UDP-GlcUA and UDP-GlcNAc have a major effect on HA synthesis. In 1995, Nakamura et al. discovered that 4-methylumbelliferone (4-MU), a coumarin derivative not only suppresses UDP-GlcUA activity, but also the mRNA levels of HAS2 and HAS3, which leads to reduced HA synthesis (Nakamura et al., 1995). This suppression has been reported in numerous cell lines such as mesothelial cells (Rilla et al., 2008), keratinocytes (Rilla et al., 2004), skin fibroblasts (Nakamura et al., 1997) and melanoma cells (Kudo et al., 2004). In keratinocytes, it was observed that a C-2 epimer of glucose, mannose, aids in the decline of cellular UDP-GlcNAc, leading to downregulation of HA synthesis (Jokela et al., 2008). It has been suggested that mannose does not affect the transcriptional regulation of GFAT1 or 2, but instead reduces the UDP-HexNAc pool (i.e. the combination of UDP-GalNAc and UDP-GlcNAc), by inhibiting the enzymatic activity of GNPDA (Çayli et al., 1999; Jokela et al., 2008). While 4-MU and mannose inhibit HA synthesis by reducing the subcellular levels of UDP-sugars, Marshall et al. reported that addition of glucosamine increased the levels of UDP-GlcNAc by bypassing the rate limiting step in HBP (Marshall, Nadeau and Yamasaki, 2005; Marshall, Yamasaki and Okuyama, 2005). Apart from glucosamine, an increase in the levels of UDP-GlcNAc has been observed after overexpression of GFAT in vascular smooth muscle and mesangial cells (Schleicher and Weigert, 2000). In accordance, in keratinocytes, a reduction of GFAT enzymes by siRNA reduces UDP-GlcNAc levels and HA levels (Oikari et al., 2016). Pitsillides et al. have shown that UDP-glucose dehydrogenase enzyme activity (UGDH) correlates with HA production (Pitsillides et al., 1993).

### 2.1.5 Hyaluronidases

The amount of HA synthesis is closely controlled as discussed above, but HA catabolism is also important for the maintenance of embryonic development, regeneration, tissue homeostasis and wound healing (Stern and Jedrzejas, 2008).
The degradation rate of HA varies depending on the tissue: in cartilage, it is 3 weeks (Morales and Hascall, 1988), in skin, one day (Tammi et al., 1991) and in plasma, only 2.5-4.5 minutes (Fraser et al., 1981). Hyaluronidases (HYAL) and lysosomal β-exoglycosidases are the enzymes responsible for HA catabolism (Stern, 2003). Hyaluronidase enzymes HYAL1-4 and PH-20 undertake the endolytic cleavage of HA polymers (Stern, 2005). Hyaluronidases are classified in vertebrates, bacteria, leeches and crustaceans based on the substrate specificity and product obtained. The human genome contains 6 HYAL genes, of which HYAL1-3 are located in chromosome 3p21.3 and HYAL4, Sperm adhesion molecule 1 (SPAM1/PH-20) and hyaluronidase pseudogene 1 (PHYAL1) in chromosome 7q31.3 (Csóka, Scherer and Stern, 1999; Stern, 2005; Stern and Jedrzejas, 2006). In mammals, HA is degraded by HYALs, which act by cleaving the β-1,4-glycosidic bond, yielding tetrasaccharides as end products (Stern, 2003). The main hyaluronidase found in plasma and urine is an acid pH-active lysosomal enzyme HYAL1 (Frost et al., 1997; Csóka, Scherer and Stern, 1999). HYAL2 is also an acid-active enzyme positioned in the plasma membrane with a GPI anchor and mainly expressed in the somatic tissues (Lepperdinger, Strobl and Kreil, 1998; Stern, 2004). According to the current hypothesis, HA is initially degraded by HYAL2 into fragments around 20 kDa in size followed by degradation into tetra- or hexasaccharides by HYAL1 in the intracellular vesicles. The oligosaccharides are further broken down to monosaccharides by lysosomal exoglycosidases (β-N-acetylglucosaminidases and β-glucuronidase) (Stern, 2003). HYAL3 is largely prevalent in the bone marrow and testis, but it can also be found spread throughout the human body (Csóka, Scherer and Stern, 1999; Csoka, Frost and Stern, 2001). While mice with HYAL1 knockout displayed an accumulation of HA in their joints leading to osteoarthritis, there was no accumulation of HA when HYAL3 was knocked-out (Hemming et al., 2008). HYAL4 is a chondroitin sulfate hydrolase involved in cleaving galactosaminidic linkages; it is found in the skeletal muscle and placenta (Stern, 2003). KIAA1199 is a new hyaluronidase-like enzyme that has been detected in synovial fibroblasts to be involved in degradation of HA. Additionally, KIAA1199 is involved in EMT by binding to and initiating EGFR signaling and in promoting glycogen breakdown, an essential step in cancer cell survival (Yoshida et al., 2013). Recently TMEM2 (transmembrane protein 2) has been identified as a cell surface hyaluronidase that cleaves the extracellular HA (Yamamoto et al., 2017; Yamaguchi et al., 2019). In addition to HYAL enzymes, reactive oxygen species (ROS) also play an important part in HA degradation (Šoltés et al., 2006).
2.1.6 Biological functions of hyaluronan

Hyaluronan is a ubiquitous extracellular matrix molecule. In addition to acting as a space filler, its molecular properties influence the tissue’s physical and hydration properties. Furthermore, HA interacts with many cell surface receptors and extracellular molecules thus modifying the cell’s behavior (Toole, 2000). The formation of the HA coat was first described in the 1970’s by Clarris and Fraser, when they found in a red blood cell exclusion test, where red blood cells were excluded in the peripheral area of cultured cells secreting HA and proteoglycans (Clarris and Fraser, 1968). Although many cell types like chondrocytes, bone-marrow derived mesenchymal stem cells and vascular smooth muscle cells produce an endogenous HA coat, a genetically modified overexpression of HASs induces the formation of pericellular HA coats (Heldin and Pertoft, 1993; Knudson and Knudson, 1993; Rilla et al., 2008). This HA coating on and between cells regulates various biological functions such as tissue homeostasis, wound healing, proliferation, regeneration and inflammation (Toole, 2000; Tammi et al., 2008, 2011). In this chapter, some of these processes will be discussed in detail.

Hyaluronan in proliferation

The impact of HA on cell cycle regulation and proliferation depends on its molecular mass, quantity and the cell type. HA accumulation during limb development is an important regulator of the proliferation and migration of cells. For example, HA accumulates in the cleavage furrow of mitotic keratinocytes (Tammi et al., 1991; Li et al., 2007). In rat mesangial cells, cyclin D3 regulates HA synthesis (Ren, Hascall and Wang, 2009) while in HepG2 cells, HA synthesis and cyclin D1 expression are elevated if HAS2 is upregulated due to the overexpression of HABP1/P-32, a HA binding protein, leading to increased cell proliferation (Kaul et al., 2012). Some studies have shown that the molecular mass of HA has an effect on proliferation; LMW HA increased cyclin D1 via HA-CD44 interaction, while HMW HA inhibited cyclin D1 expression (Kothapalli et al., 2008). A correlation between cell proliferation and the amount of HA content has been observed in many cells. For example, in epithelial cells (normal, malignant and hyperplastic) (Damodarasamy et al., 2015), human fibroblasts (normal and transformed) (Matuoka, Namba and Mitsui, 1987), mesothelial (Teder, Versnel and Heldin, 1996) and mesangial cells (Mahadevan et al., 1996), the proliferation rate of cells correlated with increased HA levels. In some cases, an addition of exogenous HA either induced a decrease in the proliferation rate, as in endothelial, flexor tendon, synovial and astrocyte cells (Goldberg and Toole, 1987; West and Kumar, 1989; Struve et al., 2005; Yagi et al., 2010), or increased the proliferation as has been
observed in melanoma cells, ovarian cancer cells and fibroblasts (Yoneda et al., 1988; Bourguignon et al., 1997; Thomas et al., 2001). Furthermore, manipulation of HA synthesis and synthase activity exerts an impact on the proliferation of the cells. Inhibiting HA synthesis by treatment with 4-MU decreases the proliferation rate in human aortic smooth muscle cells (Vigetti et al., 2009b) and rat keratinocytes (Rilla et al., 2004). The ability of HA to induce cell proliferation is impaired when its ligand binding activity with CD44 was inhibited with a CD44 blocking antibody (Thomas et al., 2001), with an antisense transgene (Kaya et al., 1997) or by treatment with oligosaccharides (Evanko, Angello and Wight, 1999).

The overexpression of HAS3 in melanoma cells decreases their proliferation (Takabe et al., 2015a), while an opposite effect i.e. an increase in proliferation, is seen in prostate cancer cells (Liu et al., 2001). In human osteosarcoma cells, inhibition of HAS2 activity decreases cell growth (Chao and Spicer, 2005) and in HYAL2 negative glioma cells, HAS2 overexpression decreases the proliferation of the cells (Enegd et al., 2002). A collaboration between growth factor receptors and HA receptors has been shown to regulate cellular behavior. For example, in osteoblastic cells, proliferation and differentiation are induced through ERK1/2 as a result of RHAMM overexpression (Hatano et al., 2011). The interaction of HA with RHAMM is MAPK dependent and also induces the proliferation in bladder smooth muscle cells (Aitken and Bägl, 2001). HA is involved in interaction between EGFR and CD44 that leads to the activation of ERK kinase pathway by promoting cell division and proliferation (Brecht et al., 1986; Meran et al., 2011).

**Hyaluronan in epithelial to mesenchymal transition**

Many biological process like embryogenesis, inflammation, regeneration, wound healing and pathological situations such as cancer, neoplasia and fibrosis show evidence of a crucial process “Epithelial to mesenchymal transition (EMT)” (Craene and Berx, 2013). During cancer progression, EMT occurs i.e. the cells lose their epithelial traits such as cell-cell adhesion and polarity, and gain mesenchymal properties like increased motility and adopt a fibroblast-like morphology (Greenburg and Hay, 1982). They also lose the expression of epithelial marker E-cadherin while gaining the expression of mesenchymal markers like vimentin, N-cadherin and fibronectin. HA plays a significant role in EMT during the above-mentioned processes (Lee and Herlyn, 2007). Epicardial cells of the zebrafish express high levels of HA and HA mediated motility receptor (Hmmr) while undergoing EMT. This occurs when the heart is going through regeneration mechanisms, signifying the importance of HA and EMT (Missinato et al., 2015).
Increased HA synthesis and CD44 expression are observed when rat mesothelial cells undergo EMT induced by wound healing (Koistinen et al., 2017). In hepatocellular and prostate cancer, the HA receptor CD44 is associated with the occurrence of neoplastic EMT (Mima et al., 2013; Shang et al., 2015). Numerous publications have demonstrated that TGF-β is one of the stimulants for EMT related cell motility and morphogenesis. HA is a notable downstream signaling molecule of the TGF-β-mediated pathway (Sengupta et al., 2013; Chanmee et al., 2014; Brockhausen et al., 2015). In breast and lung cancer, HAS1-3 expression and HA synthesis are induced by TGF-β1, which paves the way for the EGFR-CD44 interaction resulting in EMT (Li et al., 2015). In NMuMG mammary epithelial cells, EMT is induced by TGF-β; the process is dependent on HAS2 expression leading to increased HA synthesis (Porsch et al., 2013). Phenotypical changes resembling EMT occur in human epithelial and madine darby kidney cells when HAS2 is overexpressed (Zoltan-Jones et al., 2003). In normal and cancer epithelial cells, HA-mediated EMT is believed to be stimulated by numerous cytokines and growth factors (Mulshine, Chow and Tauler, 2010; Takahashi et al., 2010).

**Hyaluronan in multidrug resistance**

The ability of cancer cells to acquire resistance to anticancer therapies could be due to the occurrence of multidrug resistance and CD44 and HA have a role in this phenomenon (Misra, Ghatak and Toole, 2005); treatment with hyaluronidases helps chemotherapeutic agents to perform more effectively (Baumgartner et al., 1998). ABC transporter proteins, multidrug resistance protein 2 (MRP2) and multidrug resistance transporter 1 (MDR1) are typical multidrug resistance proteins which can be activated by HA (Guan et al., 2015; Moitra, 2015). Head and neck squamous cell carcinoma exhibited drug resistance due to exogenously added HA (Wang and Bourguignon, 2006). It has been shown that when HA interacts with oncogenic proteins with activating mutations and gene amplification or when there is an altered regulation of tyrosine kinase receptors, which is associated with HA signaling, there is an increased possibility of enhanced tumor cell growth and resistance to therapies (Toole and Slomiany, 2008). HA production is enhanced with increased expression of EMMPRIN (extracellular matrix metalloproteinase inducer) thereby stimulating multidrug resistance (Misra et al., 2003). Drug resistance correlates with an increase in HA levels not only during platinum-based chemotherapy given to ovarian cancer patients but also in cultured ovarian cells. In these cells, along with the increase in the levels of HA and ABCC2, an increase in the amount of CD44 was also observed (Ricciardelli et al., 2013). In addition, HA-mediated CD44 signaling stimulated drug resistance to doxorubicin in cultured
human mesenchymal stem cells (Liu et al., 2009). Although there are many cases where HA promotes drug resistance, one intriguing finding is that chemoresistance of cancer cells could be inhibited by LMW HA oligosaccharides (Misra et al., 2003).

### 2.2 HYALURONAN AND CANCER

In 1924, Otto Warburg described a metabolic switch in cancer cells that allows them to derive energy from glycolysis rather than oxidative phosphorylation; this accounts for the active nutrient supply demanded by cancer cells to meet their enhanced proliferation rate (Warburg, 1924). The Warburg effect is considered as a universal property of cancer cells i.e. the rate of glycolysis of the cells is increased.

Due to this phenomenon, the production of lactate is increased leading to pericellular acidification that promotes cell metastasis, invasion and drug resistance (Stern, 2009b). In numerous cancer cell types, their metastatic and aggressive behavior has been correlated with the production of HA (Kimata et al., 1983; Van Muijen et al., 1995).

The concentration of HA in each cancer stage, type and grade varies in the surrounding stroma and parenchyma (Tammi et al., 2008). The favorable niche provided for tumor growth is obtained through a HA-rich matrix (Jacobson et al., 2000). The matrix formation occurs due to the signaling events occurring between stromal and cancer cells by growth factors such as PDGF, EGF and KGF (Karvinen et al., 2003). HA functions as a mitogen in proliferating breast cancer cells, as it has been shown that HAS2 inhibition and a lack of HA synthesis leads to cell cycle arrest at G₀-G₁ phase (Udabage et al., 2005). Epithelial to mesenchymal transition was observed in mice due to increased levels of HA and HAS2 expression (Itano et al., 2002). PDGF produced by melanoma cells increases HAS2 expression in stromal fibroblasts evoking enhanced HA synthesis, evidence of the interaction between stroma and tumor cells (Pasonen-Seppänen et al., 2012; Willenberg et al., 2012). Histochemical stainings of HA in skin tumors SCC (squamous cell carcinoma) or melanoma revealed a decrease during tumor progression, which also correlated with the recurrence in melanoma and poor prognosis in SCC (Karjalainen et al., 2000; S. Karvinen et al., 2003; Kosunen et al., 2004; Poukka et al., 2016). However, in gastric, breast and colon malignancies, poor prognosis and cancer recurrence have been connected with high levels of cell associated HA (Ropponen et al., 1998; Setälä et al., 1999; Auvinen et al., 2000; Köbel et al., 2004). The stromal cells of breast cancer have high HA levels and HASs, and these phenomena have been associated with
the poor survival of the patient (Auvinen et al., 2014). Hence HA could be used as a prognostic marker in certain cancers (Tammi et al., 2008; Sironen et al., 2011).

Figure 2: Interaction of HA with its partner molecules and the functions exerted by HA after interaction. EMT = Epithelial to Mesenchymal transition.

HA shows an increase during the progression from benign skin lesions to localized melanoma and decreases in invasive and metastatic melanoma (Karjalainen et al., 2000). It has been observed that the levels of HAS1-3 and CD44 correlated directly with that of HA in various phases of melanoma. In cases of invasive and metastatic melanoma, the levels of HYAL2 expression are increased and thus there is an inverse correlation with the level of HA (Siiskonen et al., 2013). In squamous cell carcinoma of mouth, lung, esophagus and larynx cancer, well differentiated and poorly differentiated tissues exhibit an increase in CD44 staining and a decrease in HA positivity (Wang et al., 1996; Pirinen et al., 1998; Hirvikoski et al., 1999; S. Karvinen et al., 2003; Kosunen et al., 2004). A study conducted in a rat colon cancer model, revealed an elevated rate of tumor growth in association with HAS2 overexpression (Jacobson et al., 2002). Accumulation of HA due to HAS3
overexpression was associated with a loss of cancer cell adhesion in pancreatic cancer (Kultti et al., 2014b). Colon cancer cells exhibited a reduction in anchorage-independent growth when HAS3 expression was silenced by exposure to anti-sense oligonucleotides (Bullard et al., 2003).

The cellular responses and cancer progression are also influenced by the molecular size of HA. The invasive potential of breast cancer cells in vitro is promoted by low molecular weight (LMW) HA, even when there are high levels of tumor associated HA (Sapudom et al., 2017). Interference with the HA-CD44 interaction by an HA oligosaccharide (HA12) inhibited the subcutaneous melanoma formation while lymph node metastasis of melanoma was induced by LMW HA in mice (Zeng et al., 1998; Du et al., 2016). A LMW, 35 kDa HA, decreased E-cadherin expression and promoted the invasive characteristics of breast cancer cells (Zhao et al., 2017), while a CD44 dependent cell proliferation was induced in melanoma by a 30-50 kDa HA (Sapudom et al., 2017). Tian et al., suggested that in naked mole rats, high molecular weight (HMW) HA prevented cancer development (Tian et al., 2013). The presence of LMW HA has been linked to high expression of HYAL-1 and -2 in breast cancer cells (in vitro) with the same result being observed in the serum of breast cancer patient samples (Wu et al., 2015). In a normal tissue, regeneration and wound healing by free radicals and LMW-HA initiate immune cell recruitment, ECM component deposition, inflammation and epithelial cell migration. In coordination with HA receptors such as CD44, RHAMM and TLR2 and 4, HA mediate the above mentioned functions. These tightly regulated processes of tissue regeneration and wound healing associated with HA production and fragmentation are hijacked by tumor cells to drive and maintain malignancy (Liu et al., 2019). Tumor progression and wound repair have complex but common biological processes. They share molecular mechanisms that control cell survival, migration, proliferation and invasion (Tolg et al., 2014).

2.2.1 Melanoma

The prognosis of patients has a strong correlation with the magnitude of HA accumulation in the epithelial or the stromal cells in cancers such as colon, ovarian, breast, gastric and prostrate. While this pattern of positive correlation between HA content and prognosis occurs in cells and stroma secreting less endogenous HA, stratified epithelia endogenously producing high amount of HA with a HA rich stroma does not have a positive correlation. Such cancers like melanoma and squamous cell carcinoma of skin, esophagus, mouth and larynx have a negative
correlation between HA content and patient prognosis. This could be due to enhanced turnover of HA for malignant progression and invasion into surrounding environment (Tammi et al., 2008). The HA content during melanomagenesis increased in the progression of stages from benign nevi, dysplastic nevi to in situ melanoma, but the content is declined in invasive melanoma. An increase in HYAL2 levels could be the reason behind the abrupt change in HA levels in the invasive melanoma stage. During melanoma progression, the HA content appears to decline during metastases and relapse and is associated with a loss of HAS1 and 2 (Siiskonen et al., 2013; Poukka et al., 2016). Increased cellular migration and production of the pericellular HA coat are observed in metastatic melanoma cells transfected with HAS 1 and 2 (Ichikawa et al., 1999). The invasion propensity of BRO melanoma cells (cells derived from primary melanoma) is enhanced in the presence of immobilized LMW HA and their proliferation rate is increased due to the presence of soluble LMW HA. Both increased proliferation and invasion characteristics are CD44 dependent (Sapudom et al., 2017). While there are numerous studies on HA in melanoma, the exact role of HA in melanoma progression still remains unresolved.

### 2.2.2 Mesenchymal stem cells

Friedenstein and Petrakova were the first investigators to isolate mesenchymal stem cells (MSC) from the bone marrow’s stromal compartment (Friedenstein, Piatetzky-Shapiro and Petrakova, 1966). MSCs are multipotent progenitor cells that can be differentiated into multiple cell types such as adipocytes, chondrocytes and osteoblasts. MSCs’ self-renewal, reparative and plastic nature allows them to be used for tissue engineering as well as in therapeutic applications (Barry and Murphy, 2004). Over the years, it has become evident that MSCs are involved in tumor growth, metastasis and angiogenesis through intercellular communication. Even though the underlying mechanism behind MSCs’ action is unclear, the potential effectors could be the secretion of bioactive molecules, which cause lineage reprogramming en route to mesenchymal cells, and immunosuppressive action of the MSCs (Du et al., 2014). The CD44-HA interaction has also been shown to be responsible for the migration of MSCs from the bloodstream to the tissues during their regeneration and during graft fibrosis (Zhu et al., 2006). Inflammation in the cartilage leads to HA accumulation which attracts the MSCs to the injured area (P.E. et al., 2001). The natural abundance of HA in the ECM and its role in proliferation, migration, differentiation, embryogenesis and drug resistance make it a suitable molecule to allow the stem cells to form a niche matrix. All isoforms of
HASs are expressed at high levels by MSCs. The CD44 receptor on the cell surface harbors the produced HA, leading to the development of an extensive HA coat around the MSCs (Qu et al., 2014). The paracrine signaling of the MSCs is partly due to the EVs that they release, which could explain their (EVs) anti-tumor activity (Rani et al., 2015). There are reports highlighting the involvement of MSC-derived EVs in reducing tumor cell apoptosis in bladder cancer (Wu et al., 2013), and also necrosis in ovarian cancer cells (Bruno et al., 2013) and reversing chemo-resistance in glioblastoma (Munoz et al., 2013). These recent findings suggest that HA and EVs secreted by MSCs may have potential applications in the field of regenerative medicine and cancer therapies.

2.3 EXTRACELLULAR VESICLES

Extracellular vesicles (EVs) are spherical particles surrounded by a phospholipid bilayer. They are released from both prokaryotic and eukaryotic cells and act as important mediators in cell-cell communication. In most cases, EVs reflect the molecular composition of the original cells, carrying signaling molecules, coagulation factors and genetic information. They are also believed to be responsible for cellular waste management (Nieuwland and Sturk, 2010). In addition to regulating various biological processes in both health and disease, they can also serve as prognostic factors, biomarkers and tools of therapy (van der Pol et al., 2012). The lipid membrane enclosing the EVs is enriched with annexin, cholesterol, sphingomyelin and glycosphingolipids; these confer protection and stability for the cargoes carried by the EVs into the extracellular space (Maas, Breakefield and Weaver, 2017). EVs were initially identified in the 1940s from human plasma supernatant. The pellet obtained after high speed centrifugation contained the plasma’s clotting factor, since the supernatant did not contain this factor, it prolonged the clotting time (Chargaff and West, 1946). Wolf et al., termed the vesicles originating from the platelet in this subcellular fraction as “platelet dust” and these vesicles ranged between 20 to 50 nm in diameter (Wolf, 1967). Though vesicles were initially considered to regulate basic cellular functions, it was not until the 1980s that their role in carrying the molecules or soluble factors from the cells was studied. The 1980’s witnessed multiple milestones in vesicle biology. In 1983, the formation of multivesicular bodies (MVBs) with vesicles inside them was found and the vesicles released from the MVBs into the extracellular environment were considered as cellular waste (Harding and Stahl, 1983; Pan and Johnstone, 1983). Trams et al., found that microvesicles were released from neoplastic cells (Trams et al., 1981) and in 1987, the vesicles isolated from sheep
reticulocytes were named “exosomes” (Johnstone et al., 1987). The past decade has seen an in-depth exploration of the EV field and revealed the fact that EVs of varying sizes are released via plasma membrane budding and by the endosomal pathway. These EVs are referred to by a variety of names like microparticles, exosomes, ectosomes and oncosomes (Maas, Breakefield and Weaver, 2017). The subpopulations of EVs are defined by their origin and existence either inside or outside of the cells. In addition to the above properties, EVs are also classified based on size, density and cargo (György et al., 2011). Irrespective of the differences in composition and biogenesis, the terminology “EV” embodies all categories of membrane vesicles released into the extracellular environment (Colombo, Raposo and Théry, 2014).

EVs are required not only to trigger pathological events but also in intercellular communication in healthy individuals. They contribute to the maintenance and regulation of reproduction, coagulation, tissue repair, embryonic development, immune modulation and angiogenesis (Yáñez-Mó et al., 2015). During pathological events like cancer, the autocrine and paracrine functions exhibited by EVs contribute to many phenomena, e.g. pre-metastatic niche formation, epithelial to mesenchymal transition, immunosuppression, invasion, unregulated proliferation and obstructing cell death (Azmi, Bao and Sarkar, 2013; Maas, Breakefield and Weaver, 2017).

### 2.3.1 Biogenesis and secretion of EVs

The diversity in the composition, size and abundance of EVs is not only based on the parental cells that secrete these structures, but also the various sorting mechanisms and biogenetic pathways through which the EVs are released into the extracellular space contribute to their diversity. EVs have been classified into 3 major categories as exosomes (30-150 nm size range), microvesicles (100-1000 nm size range) and apoptotic bodies (50-5000 nm size range) (Anand et al., 2019). The differentiation is based on their intracellular site of origin, distinct molecular and structural properties, which not only highlights the diversity of biogenetic pathways but also carries information about the condition of the parental cells (Théry, Ostrowski and Segura, 2009a). Exosomes are released from the multivesicular bodies through the endosomal pathway (Gan and Gould, 2011), while microvesicles are released either by direct budding from the plasma membrane or from the ends of microvillus-like plasma membrane protrusions (Rilla et al., 2014). Various sizes of apoptotic bodies dissociate from cells undergoing
apoptosis; these are difficult to differentiate from other EVs except that they might carry more genomic DNA (Tkach and Théry, 2016). As a point of interest, HA synthesis is associated with increased secretion of EVs in cells with high endogenous or overexpression of HAS. Though it is not clear what type of EVs are associated with HA synthesis in particular, EVs in the size less than 200-300 nm are frequently observed (Arasu et al., 2019).

The occurrence of a lipid curvature at the plasma membrane leads to an outward budding vesicle (microvesicles) or an inward budding vesicle for exosome formation in the endocytic system (Maas, Breakefield and Weaver, 2017). The endosomal sorting complex required for the transport (ESCRT) machinery comprises ESCRT-0, I, II, III, TSG101 and ALIX (ALG-2 interacting protein X) (Hurley and Hanson, 2010). The intraluminal vesicle (ILV) formation is controlled by the ESCRT complexes I and II that regulate the invagination of the late endosomes, leading to the formation of ~40-100 nm ILVs within the MVBs (multivesicular bodies). The cytosolic components become engulfed, and transmembrane and peripheral proteins are incorporated during the formation of the ILV (Théry, 2011; Christ et al., 2017). The MVBs are either destined for lysosomal degradation (Johnstone et al., 1987) or merged with the plasma membrane before being released as exosomes into the extracellular environment (Théry, Zitvogel and Amigorena, 2002). Some of the MVBs are diverted from lysosomal degradation due to their distinctive signature which occurs as a result of the Alix protein interacting with ESCRT binding syntenin (Hurley and Odorizzi, 2012). Secretion of exosomes is regulated by several molecular regulators such as Cortactin (a cortical actin regulator) (Sinha et al., 2016) as well as the molecules involved in docking of MVBs like Rab27a, Rab27b, RapA and Rab35 proteins (Hsu et al., 2010; Ostrowski et al., 2010; Hyenne et al., 2015) and synaptotagmin (a fusion regulator) (Hoshino et al., 2013). While a few alternative pathways such as the induction of lipid curvature via ceramide synthesis (Trajkovic et al., 2008; Kajimoto et al., 2013) and sorting of specific proteins facilitated by tetraspanin have been shown in exosome biogenesis, the predominant mechanism invariably involves the endosomal pathway (Theos et al., 2006; Fast et al., 2017). The alternative mechanisms seem to address either the formation or sorting of the ILV but not both and are not totally independent processes as they might still merge with the ESCRT machinery. All the studies addressing the biogenesis of exosomes have been conducted exploiting knock-down approaches and it has been speculated that a clearer picture of the biogenesis would be obtained if gene editing techniques could be applied in these studies (Maas, Breakefield and Weaver, 2017).
2.3.2 Molecular composition of EVs

The EV cargo is highly heterogenous owing to its ability to carry nucleic acids, lipids, proteins, surface molecules and other metabolites. Even though the EV cargo has some of the characteristics of the parental cells from which they are released, they also consist of components incorporated due to a regulated sorting mechanism and this part has remained unresolved (Pathan et al., 2019). Liquid biological samples contain EV subpopulations released from various cell types, making them difficult to differentiate as there is no isolation method to obtain pure subpopulations (Colombo, Raposo and Théry, 2014). There have been studies conducted revealing donor cell specific EV signatures (Muturi et al., 2013) which extend up to specific EV signature for cellular transformation and differentiation (Garnier et al., 2013). The common EV markers used are present in all EV subsets.

Figure 3: A graphical representation of the EV biogenesis. (i) Microvesicles: a lipid curvature at the plasma membrane leads to an outward budding vesicle. They bud directly from the plasma membrane or from the tips of plasma membrane protrusions. (ii) Exosomes: an inward budding vesicle is initiated by endocytosis. Once the process is initiated early endosomes are formed and then multi-vesicular bodies (MVB) or late endosomes are formed. MVBs either fuse with the plasma membrane releasing the exosomes into the ECM or are targeted towards lysosomes for degradation. (iii) Apoptotic bodies: Once the cells undergo nuclear condensation, fragmentation and cell shrinkage it is signaled for programmed cell death. Apoptotic bodies are EVs generated during this process.
and there is no specific marker to differentiate between each EV subset (Aatonen et al., 2014; Clark et al., 2015; Keerthikumar et al., 2015; Minciacchi et al., 2015; Xu et al., 2015). Kowal et al. have described that EVs derived from all biological sources can be categorized as: 1) low speed precipitates, called large EVs, 2) medium sized EVs and 3) smaller EVs or sEVs obtained after high speed centrifugation. The sEVs can be further categorized as: a) bonafide exosomes rich in endosome markers and tetraspanins like CD63, CD9 and CD81, b) sEVs that are CD9-positive but negative for CD63 and CD81. These sEVs are associated with the plasma membrane and early endosomes, c) sEVs negative for all the tetraspanins and d) sEVs that are abundantly present in ECM and contain serum derived factors; these sEVs are usually not associated with the endosomal route of vesicles (Kowal et al., 2016).

The pathological and physiological state of the parental cells, the pathways and stimuli leading to the formation/release of EVs are the main conditions that control the nature and content of the EV cargo (Minciacchi, Freeman and Di Vizio, 2015). Due to these characteristics of the EVs, one priority has emerged, i.e. clarifying the cargo of EVs as they may represent potential biomarkers (Anand et al., 2019). Théry et al. confirmed the presence of many membrane and cytosolic proteins in exosomes collected from dendritic cells using proteomic analysis (Théry et al., 1999). Based on this study, many groups have studied EVs from a variety of sources, to identify certain common EV markers. Some of the common EV markers in the protein category include cytosolic proteins such as heteromeric G protein Gi2α, annexin II, flotillin, Rabs, tetraspanins (CD9, CD63, CD81), MHC class II complex, ESCRT proteins, HSP (heat shock proteins) 70, 90, TSG101 and Alix (Mathivanan, Ji and Simpson, 2010; Witwer et al., 2013; Anand et al., 2019). Common EV markers in the lipid category enriched in the EVs include ceramide, phosphatidylinerine, sphingomyelin, gangliosides, cholesterol and glycosphingolipids (Zaborowski et al., 2015; Kalra, Drummen and Mathivanan, 2016). The presence of RNA in EVs was detected in 2007; this was a ground-breaking discovery as the authors showed that mRNAs and miRNAs were carried by the EVs and the target cells receiving the RNA were able to efficiently translate the message that it carried (Ratajczak et al., 2006; Valadi et al., 2007). An unbiased deep sequencing of RNA in EVs revealed that a variety of other types of small non-coding RNAs like vault RNA, small interfering RNA, repeat sequences, long non-coding RNA, structural RNA, tRNA fragments and Y RNA were also present in EVs (Bellingham, Coleman and Hill, 2012; Nolte’T Hoen et al., 2012). A recent finding has been the identification of the presence of DNA molecules such as mitochondrial DNA (Guescini et al., 2010), single-stranded DNA (Balaj et al., 2011) and double stranded DNA (Waldenström et al., 2012; Cai et
al., 2013) in EVs. The mechanism behind sorting of nucleic acids into EVs has still not been clarified. Online databases such as vesiclepedia (Kalra et al., 2012), EcoCarta (Mathivanan et al., 2012) and EVpedia (Kim et al., 2015) provide access to compiled data on the components of the EVs from the various studies that have been reported.

2.3.3 EVs role in cell to cell communication

Cell-to-cell communication can occur either through autocrine, paracrine or endocrine signaling and is a crucial regulator for various pathological and biological processes. Along with signaling molecules such as growth factors, hormones, lipids and cytokines, EVs also act as signalosomes. The protein composition influences their biodistribution, as the receptors and ligands exposed on their surface determine the binding of the EVs either to the ECM or to some target cell (Yáñez-Mó et al., 2015). The EVs released into the circulation are either eliminated from the body in the bodily fluids like urine or are internalized and thereby can influence the properties of a target cell via the cargo they carry (Ratajczak et al., 2006; Valadi et al., 2007; Al-Nedawi et al., 2008). Detection of EV fusion with the plasma membrane of live cells has been challenging, given the limitations in the resolution of light microscopes. Recent studies using EVs carrying GFP-labeled transmembrane proteins have allowed a visualization of EV interaction with the plasma membrane of the recipient cells using a technique called correlative light and electron microscopy (CLEM) (Arasu et al., 2019). There was a visible increase in the fluorescence of the recipient cells when EVs labelled with lipophilic dye R18 were added (Montecalvo et al., 2012).

While the mode of interaction of EVs with the target cells has been controversial, several basic routes have been found 1) through ligand-receptor interactions (Yáñez-Mó et al., 2015), 2) direct fusion with the plasma membrane (Del Conde et al., 2005; Parolini et al., 2009), 3) through microtubes (Osswald et al., 2016) and ‘tunneling’ nanotubes (Connor et al., 2015) or 4) internalization as intact EVs followed by release of the contents inside the cells. Apart from the above mentioned mechanisms, EV internalization has been suggested to occur through multiple other ways such as via caveolin mediated endocytosis (Nanbo et al., 2013; Svensson et al., 2013), phagocytosis (Feng et al., 2010), clathrin mediated endocytosis (Escrevente et al., 2011) and macropinocytosis (Fitzner et al., 2011). Though it is not very clear at the moment, multiple routes of EV interaction with the target cells could be due to their surface composition. The internalization of EVs by specific interaction mechanisms has been confirmed by the identification of proteins like integrins,
immunoglobulins and tetraspanins present in the EVs (Mulcahy, Pink and Carter, 2014). Once internalized, the EVs may be fused with the endosomal delimiting membrane or be directed to lysosomes for degradation (Raposo and Stoorvogel, 2013). Some of the phenotypic changes acquired by the recipient cells are possibly mediated by receptor ligand interaction rather than by internalizing the EVs (Mulcahy, Pink and Carter, 2014; Yáñez-Mó et al., 2015). The direct fusion of the EVs with the recipient cells can be considered as a passive occurrence. During direct fusion, a continuous structure is formed after the membrane of the cells and EVs merge together (Minciacchi, Freeman and Di Vizio, 2015). Although many of the details on EV communication with cells or organs are now known, the key regulatory features driving the binding and release of EV contents to the recipient cells have remained elusive.

2.4 EVs IN CANCER

The malignancy of a tumor is based on both the cancerous cells and the surrounding tumor microenvironment. For the initiation and progression of cancer, the cells and the microenvironment interact with each other via various secretory molecules such as cytokines, growth factors, chemokines, ECM binding integrins and the newest mode of identified interacting machinery i.e. EVs (Naito et al., 2017). The content and population of the EVs released from the cancer cells are very different from the EVs released from normal cells. It has been speculated that these contents can act as a snapshot of the tumorigenic cells and a hint at their behaviour (Tkach and Théry, 2016). The cancer cell derived EVs educate the surrounding stroma and cells so that they acquire many of the hallmarks of cancer. This can be combatted by EVs from healthy cells conveying tumor suppressive properties. Understanding the pivotal role of EVs in cancer biology can help to understand tumor progression and obtain some valuable prognostic and therapeutic markers (Xie et al., 2019). Premetastatic niches are tissue sites “prepared” by factors released from primary tumor cells making the site suitable to allow the tumor to metastasize. EVs from pancreatic ductal adenocarcinoma established a liver metastatic niche by priming the Kupffer cells in the liver via the transfer of migration inhibitory factor (MIF) (Costa-Silva et al., 2015). A premetastatic microenvironment is created due to the transformation of fibroblasts into cancer associated fibroblasts (CAFs), a process which can occur with the support of tumor derived EVs (Webber et al., 2015). EVs derived from CAFs were able to promote unregulated proliferation and metastasis in oral squamous carcinoma cells, by triggering MAPK and AKT signaling pathways. All premetastatic niche formation
Figure 4: Representation of the hallmarks of cancer and effects of cancer cell derived EVs on neighboring cells and surrounding microenvironment. The molecular composition of the EVs consists of lipids, nucleic acids, proteins and other metabolites.
are not random; some of them are formed at specific pre-meditated location. Integrins like α6β4, αvβ5 and α6β1 are secreted in the tumor exosomes, which directs them to specific tissues enabling the exosomes to fuse with the target cell and initiate pre-metastatic niche. (Hoshino et al., 2015). The EVs are able to undertake this process as they are enriched with protein MFAP5, a component from extracellular microfibrils (Principe et al., 2018). Phosphatidylserine is located on the outer layer of the EVs derived from ovarian tumor ascites fluid; this is considered to be responsible for obstructing the T cell signaling pathway. This indicates that the lipid composition is also partly responsible for the effects caused by the EVs (Kelleher et al., 2015). An unregulated proliferation and anti-apoptosis effect can be exerted on cells exposed to EVs derived from cancer cells positive for human papilloma virus (HPV). Pancreatic cells PC-1.0 secrete exosomes that carry zinc transpoter ZIP4 that when co-cultured with moderately malignant cells increase cell proliferation (Jin et al., 2018). Fatty acid oxidation (FAO) proteins are shown to hitch a ride with exosomes that are released from adipocytes, which communicate with melanoma cells to increase its migration capacity (Lazar et al., 2016).

Even though the cancer cell derived EVs are constantly subjected to complex immune surveillance, it does seem that they are able to 1) evade the attack by immune cells, 2) assist in immune tolerance and 3) escape from immune scrutiny (Théry, Ostrowski and Segura, 2009b; Greening et al., 2015). The EVs collected from melanoma cells and head and neck squamous carcinoma cells have been loaded variably with FasL. These EVs were reported to impair signaling and induce apoptosis of CD8+ T cells (Wieckowski et al., 2009). An increase in immunosuppressive adenosine was observed when Tregs, a T-cell subtype, were treated with tumor derived EVs (Muller et al., 2016). While there are a few examples showing the effect of EVs in cancer progression, there have been various studies demonstrating that EVs are able to modulate the hallmarks of cancer such as counteracting cell death, evading immune surveillance, stimulating angiogenesis, supporting proliferative signaling, triggering invasion and metastasis. The functional elements carried by the EVs which allow recipient cells to attain the hallmarks of cancer are discussed briefly below (Table 1). Even though it does seem that EVs released by cancer cells promote tumor acquiring properties, there have been also studies showing that progression of cancer can be negatively regulated by EVs. EVs released from cancer cells are packed with tumor antigens which then interact with immune cells, triggering anti-cancer immunity to kill the cancer cells (Wolfers et al., 2001; Taylor and Gerçel-Taylor, 2005).
It was reported that EVs derived from bone marrow-mesenchymal stem cells (BM-MSCs) of healthy donors carry miR-15 that helps to promote tumor suppression (Roccaro et al., 2013).

Table 1. The bioactive molecules carried by EVs and their impact on recipient cell functions.

<table>
<thead>
<tr>
<th>EV cargo</th>
<th>Donor cells</th>
<th>Recipient cells</th>
<th>Functions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β</td>
<td>Cancer cells</td>
<td>Mesenchymal Stem cells</td>
<td>Stimulating CAF phenotype, proliferation and invasion</td>
<td>(Chowdhury et al., 2015)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SMAD/TGF-β pathway activation leading to CAF phenotype</td>
<td>(Gu et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>Immune cells</td>
<td></td>
<td>Increasing immune suppression via regulatory T cells and hampering the cytotoxic functions of NK cells</td>
<td>(Clayton et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>Fibroblasts</td>
<td></td>
<td>Other cargo in this EV include FasL, IL-10 and MAGE. They together cause apoptosis of T cells carrying CD8 and induce regulatory T cell proliferation.</td>
<td>(Szajnik et al., 2010)</td>
</tr>
<tr>
<td>Integrins</td>
<td>Cancer cells</td>
<td>Cancer cells</td>
<td>Stimulates cell migration and adhesion</td>
<td>(Fedele et al., 2015a)</td>
</tr>
<tr>
<td></td>
<td>Fibroblasts</td>
<td></td>
<td>Induces cell migration, growth and increases gene expression of S100</td>
<td>(Hoshino et al., 2015)</td>
</tr>
<tr>
<td>EGFR</td>
<td>Cancer cells</td>
<td>Immune cells</td>
<td>Other cargo in this EV include syntenin-1, chondroitin sulfate proteo-glycan 4, α2-macroglobulin, lactadherin and integrin α-3 &amp; 5. The components of this EV causes maturation of macrophages from monocytes by differentiation</td>
<td>(De Vrij et al., 2015)</td>
</tr>
<tr>
<td></td>
<td>Cancer cells</td>
<td></td>
<td>A variant of EGFR (EGFRvIII) promotes support free growth and physiological changes</td>
<td>(Al-Nedawi et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>Cancer cells</td>
<td></td>
<td>mRNA of variant EGFR (EGFRvIII) induces unregulated cancer cell proliferation</td>
<td>(Skog et al., 2008)</td>
</tr>
<tr>
<td>FasL</td>
<td>Cancer cells</td>
<td>Immune cells</td>
<td>Stimulates lymphocyte apoptosis</td>
<td>(Andreola et al., 2002)</td>
</tr>
<tr>
<td><strong>TSPAN-8</strong></td>
<td><strong>Cancer cells</strong></td>
<td><strong>Endothelial cells</strong></td>
<td>Tetraspanin-8 (TSPAN-8) along with CD106 and CD49d instigate maturation, growth, vascularization and migration of endothelial cells</td>
<td>(Nazarenko et al., 2010)</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td><strong>Micro-RNAs (miR/miRNA)</strong></td>
<td><strong>Cancer cells</strong></td>
<td><strong>Endothelial cells</strong></td>
<td>miR-181c helps induce brain metastasis by disrupting the blood brain barrier permeability</td>
<td>(Tominaga et al., 2015)</td>
</tr>
<tr>
<td><strong>Cancer Cells</strong></td>
<td><strong>Cancer cells</strong></td>
<td>miR-200 family aids in triggering an epithelial to mesenchymal transition which paves the way for colony formation after metastases</td>
<td>(Le et al., 2014)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>miR-584 along with numerous other miRNAs activates p38 MAPK/JNK pathway and initiates growth of tumor</td>
<td>(Kogure et al., 2011)</td>
<td></td>
</tr>
<tr>
<td><strong>Cancer cells</strong></td>
<td><strong>Immune cells</strong></td>
<td>miR-155 expression is increased using TLR8 based mechanism by miR-21</td>
<td>(Challagundla et al., 2015)</td>
<td></td>
</tr>
<tr>
<td><strong>Immune cells</strong></td>
<td><strong>Cancer cells</strong></td>
<td>miR-155 affects TERF1 thereby rendering cancer cells with increased chemoresistance</td>
<td>(Challagundla et al., 2015)</td>
<td></td>
</tr>
<tr>
<td><strong>Cancer cells</strong></td>
<td><strong>Fibroblasts</strong></td>
<td>In fibroblasts miR-122 decreases glucose consumption</td>
<td>(Fong et al., 2015)</td>
<td></td>
</tr>
<tr>
<td><strong>Fibroblasts</strong></td>
<td><strong>Cancer cells</strong></td>
<td>EMT is activated by miR-409</td>
<td>(Josson et al., 2015)</td>
<td></td>
</tr>
<tr>
<td><strong>CD44</strong></td>
<td><strong>Cancer cells</strong></td>
<td><strong>Immune cells</strong></td>
<td>Mediates the maturation of monocytes into tumor-associated macrophages</td>
<td>(Baj-Krzyworzeka et al., 2007)</td>
</tr>
<tr>
<td><strong>SDF-1</strong></td>
<td><strong>Bone marrow stromal</strong></td>
<td><strong>Cancer cells</strong></td>
<td>The recipient cells resistance to drugs, its migration and growth is promoted by stromal cell-derived factor-1 (SDF-1) along with monocyte chemo-attractant protein-1 and</td>
<td>(Wang et al., 2014)</td>
</tr>
<tr>
<td>Cells</td>
<td>interferon-inducible protein 10</td>
<td>(Vered et al., 2015)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------------------</td>
<td>---------------------------------------------------------</td>
<td>----------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caveolin-1 Cancer cells Fibroblasts</td>
<td>Enhances differentiation of fibroblasts into CAFs</td>
<td>Caveolin-1 Cancer cells Fibroblasts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epiregulin Cancer cells Cancer cells</td>
<td>Stimulating transition of cells from epithelial to mesenchymal phenotype under E-cadherin/Gli-1 regulation</td>
<td>Yang et al., 2017</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 2.4.1 Cancer diagnosis and prognosis using EVs

The diagnosis and monitoring of cancer are major challenges and identifying new efficient biomarkers could help in the diagnosis and prognosis of the disease. In cancer biology, EVs are being studied since they possess remarkable properties. On one hand, they can educate the surrounding cells and stroma to help in tumor progression. On the other hand, EVs from non-cancerous cells repress metastases and induce apoptosis of cancer cells (Naito et al., 2017). Tumor EVs are currently being explored as cancer biomarkers due to their composition and cargo. The lipid bilayer composition of the EVs protects their contents from degradation during transit and carries bioactive molecules that can be assayed as biomarkers (Lane et al., 2018). Cancer cells release EVs into the biofluids such as urine, blood and cerebrospinal fluid making them attractive biomarkers, which can be obtained and analyzed by relatively non-invasive methods. The genetic and epigenetic changes occurring inside the cells in the tumor microenvironment make it more challenging to detect any acquired drug resistance. EVs carrying circulating free DNA (cfDNA) can be an option for monitoring the clinical response to treatments and evaluating genetic and epigenetic changes (Xie et al., 2019). EV-associated nucleic acids and membrane proteins have been shown to be a treasure-trove of biomarkers. Studies have shown EVs carrying cancer related mRNA (Skog et al., 2008), long non-coding RNA (Kogure et al., 2013), miRNA (Rabinowits et al., 2009) and proteins (Chen et al., 2017), making them attractive options as biomarkers. EVs collected from cerebrospinal fluid of glioblastoma multiforme cancer patients displayed higher levels of miR21 when compared to the EVs obtained from healthy patients (Shi et al., 2015). Nucleic acids and protein signatures have been identified in EVs collected from patients with esophageal cancer (Tanaka et al., 2013), cervical cancer (Liu et al., 2014), pancreatic cancer (Madhavan et al., 2015; Melo et al., 2015), melanoma (Peinado et al., 2012; Pfeffer et al., 2015), lung cancer (Li et al., 2011; Silva et al., 2011; Yamashita et al., 2013), breast cancer (Moon et al., 2016; Vardaki et al., 2016) and prostate cancer (Øverbye et al., 2015; Royo et al., 2016). All the markers identified in
these EVs may be able to serve as potential diagnostic markers for the respective cancers.

Some studies suggest that tumor EVs reflect the cells’ response to treatment; they may also influence the properties of certain cancers by conferring resistance by transfer of specific proteins or nucleic acids. For example, in soft tissue sarcoma, an EV carrying miR-761 conferred resistance to pazopanib (chemotherapy) (Shiozawa et al., 2018), in colon cancer, EVs carrying PTEN promoted cetuximab resistance (anti-EGFR) (Zhang et al., 2017) and in breast cancer, the presence of HER2 in the EVs bound to the drug trastuzumab (anti-HER2) which caused a weakening of the therapeutic effect (Ciravolo et al., 2012). All these reports suggest that EVs are prognostically informative and have the ability to be a disease controlling system that is non-invasive. However, in the future, clinical studies will be needed to reveal which specific miRNAs and mRNAs are carried by the tumor EVs (Lane et al., 2018). It is also worthwhile noting that there are limitations in studying EVs as biomarkers such as lack of standard protocols for EV isolation and characterization (Whiteside, 2015). Furthermore, the majority of the EV studies have been performed in in vitro cell line models and studying EVs from clinical samples is still a futuristic approach (Lane et al., 2018). EVs are like “double edged swords” which can both stimulate and repress cancer progression. Nonetheless, it can be predicted that with advances in the characterization, sample handling and experimental reproducibility, EVs will become valuable assets in cancer diagnosis and therapy.

2.5 HEDGEHOG SIGNALING PATHWAY AND CANCER

Hedgehog (HH) signaling is a conserved signaling pathway and the HH protein family controls the proliferation, growth, differentiation and survival during embryogenesis (Ruiz i Altaba, Sánchez and Dahmane, 2002). The HH pathway in mammals is activated by secreted glycoproteins: Indian (IHH), Sonic (SHH) and Desert hedgehog (DHH). The HH signaling pathway is initiated by binding of the HH ligands to their receptor PTCH1 (12-pass transmembrane receptor Patched). Under basal conditions, smothened (SMO), a G protein-coupled receptor is blocked by PTCH1. Binding of HH ligands to the PTCH1 receptor abolishes the blockade on SMO, which in turn activates zinc-finger transcription factors GLI 1-3 through signal transduction (Pietrobono et al., 2018). Upregulation of the HH pathway activates a cascade of downstream intracellular events that regulate cell survival, proliferation and differentiation. For example, the expressions of mitotic genes like CYCLIN D1, MYC and insulin-like growth factor 2 are enhanced in the
external granular layer during cerebellum development by SHH released by Purkinje cells (Wechsler-Reya and Scott, 1999). Any perturbation in the HH pathway leads to the development of numerous types of tumors such as brain, skin, pancreas, lung and stomach malignancies (Teglund and Toftgård, 2010; McMillan and Matsui, 2012; Pak and Segal, 2016). In rat kidney epithelial cells, GLI1 promoted EMT by increasing SNAIL expression (Li et al., 2006). The aggressiveness of human melanoma was directly proportional to GLI2 expression and knocking-down of GLI2 drastically decreased its invasiveness (Alexaki et al., 2010). Liu et al. have shown that in the early limb bud transcriptome (mouse), the HAS2 promoter has 2 strong GLI binding consensus sequences. It has also been shown that the transcriptional activity of HAS2 is directly controlled by SHH signaling (Liu et al., 2013). In medulloblastoma and basal cell carcinoma, treatment with antagonists targeting the HH proteins was able to inhibit tumor growth (Evangelista, Tian and De Sauvage, 2006). Some studies have shown that the cells producing HH ligands spread them to other cells via EV mediated transportation (Théron, 2012; Vyas et al., 2014). There are not many studies describing the link between HA and HH pathways. Therefore, it was deemed interesting to learn more about the relationship between HA and HH pathways, as both have important functions in basic cell biochemistry and also in cancer progression.
3 AIMS OF THE STUDY

The aims of this thesis were to investigate the factors controlling secretion of extracellular vesicles induced by HAS3 enzyme, the composition of the EVs and their role in intercellular communication. EVs secreted from HAS3 over-expressing cells were chosen, as HAS3 is more abundant on the plasma membrane than HAS1 and 2.

The definitive aims of the thesis include:

1. To investigate whether EV secretion is based on the residence time of HAS3 in the plasma membrane and whether factors such as substrate availability are able to regulate the EV secretion activity.
2. To determine if primary cells without gene manipulation, such as human mesenchymal stem cells, secrete HA-coated EVs, and to examine if this secretion is associated with HAS activity.
3. To study the composition of the HAS3-induced EVs and their ability to trigger signaling mechanisms related to cancer progression in recipient cells.
4 SUBJECT AND METHODS

4.1 MATERIALS

4.1.1 Cell lines

Table 2. Human cell lines were used in this thesis work. Specific details are explained in the original publications.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell line</th>
<th>Original Publications</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metastatic melanoma cells</td>
<td>MV3</td>
<td>I, III</td>
<td>(van Muijen et al., 1991)</td>
</tr>
<tr>
<td>Metastatic melanoma cells (with EGFP-HAS3 overexpression)</td>
<td>MV3-GFP HAS3</td>
<td>I, III</td>
<td>(Takabe et al., 2015b); Dr. Genevieve Bart, University of Eastern Finland</td>
</tr>
<tr>
<td>Melanoma cells</td>
<td>WM115</td>
<td>III</td>
<td>(Qu et al., 2014); Bone marrow samples obtained from Kuopio University Hospital</td>
</tr>
<tr>
<td>Metastatic melanoma cells (with EGFP-HAS3 overexpression)</td>
<td>C8161-GFP HAS3</td>
<td>III</td>
<td>Dr. Genevieve Bart, University of Eastern Finland</td>
</tr>
<tr>
<td>Bone marrow derived mesenchymal stem cells</td>
<td>MSC</td>
<td>II</td>
<td>(Koistinen et al., 2004)</td>
</tr>
<tr>
<td>Epidermal keratinocyte cells</td>
<td>HaCaT</td>
<td>III</td>
<td>(Boukamp et al., 1988)</td>
</tr>
</tbody>
</table>

4.1.2 Human tissue samples

The histopathological tissue specimens were acquired from Kuopio University Hospital. For stainings, 5 µm thick tissue sections from formalin-fixed and paraffin-embedded samples were used. Ethical approval was obtained from Kuopio University Hospital and the Finnish National Supervisory Authority for Welfare and Health (VALVIRA). A more detailed explanation is provided in original publication III.
4.2 METHODS

The materials and methods used are described in this chapter. Specific details are explained in the original publications.

Table 3. Methods to visualize and characterize EVs. Protocols are explained in detail in the original publications

<table>
<thead>
<tr>
<th>Method</th>
<th>Purpose</th>
<th>Original publication</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanoparticle Tracking Analysis (NTA)</td>
<td>To measure the number and concentration of EVs</td>
<td>II, III</td>
<td>Method optimized in original publications II, III</td>
</tr>
<tr>
<td>Confocal Microscopy</td>
<td>To visualize and quantify the secretion of EVs from donor cells and binding to recipient cells</td>
<td>I, II, III</td>
<td>Method optimized in original publications I-III</td>
</tr>
<tr>
<td>Transmission Electron Microscopy</td>
<td>To visualize the fine structure of EVs</td>
<td>II, III</td>
<td>Method optimized in original publications II, III</td>
</tr>
<tr>
<td>Scanning Electron Microscopy</td>
<td>To visualize the surface morphology of EVs</td>
<td>II</td>
<td>Method optimized in original publication II</td>
</tr>
</tbody>
</table>

Table 4. Methods to study UDP-sugars and HA. Protocols are explained in detail in the original publications

<table>
<thead>
<tr>
<th>Method</th>
<th>Purpose</th>
<th>Original publication</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme linked sorbent assay for HA</td>
<td>To measure HA secreted into culture medium</td>
<td>II, III</td>
<td>(Hiltunen et al., 2002)</td>
</tr>
<tr>
<td>Confocal Microscopy (Fluorescence immunohistochemistry)</td>
<td>To visualize pericellular HA coat in cells and EVs</td>
<td>II, III</td>
<td>(Rilla et al., 2008)</td>
</tr>
<tr>
<td>Anion exchange HPLC</td>
<td>To measure UDP-sugar content of cells</td>
<td>I</td>
<td>(Tomiya et al., 2001)</td>
</tr>
</tbody>
</table>

Table 5. Methods to study cell biological functions affected by EVs. Protocols are explained in detail in the original publications.
<table>
<thead>
<tr>
<th>Method</th>
<th>Purpose</th>
<th>Original publication</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell counting with hemocytometer</td>
<td>To study cell proliferation</td>
<td>III</td>
<td>Method optimized in original publications III</td>
</tr>
<tr>
<td>Type I collagen 96-well plate</td>
<td>To study cell invasion</td>
<td>III</td>
<td>Method optimized in original publications III</td>
</tr>
</tbody>
</table>

Table 6: Methods to study whole cell RNA and protein. Protocols are explained in detail in the original publications.

<table>
<thead>
<tr>
<th>Method</th>
<th>Purpose</th>
<th>Original publication</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell cycle array</td>
<td>To identify and quantitate cell cycle proteins expressed after EV treatment</td>
<td>III</td>
<td>Manufacturer’s instructions</td>
</tr>
<tr>
<td>Tandem mass spectrometry</td>
<td>To identify differentially expressed proteins after EV treatment</td>
<td>III</td>
<td>(Laakkonen et al., 2017)</td>
</tr>
<tr>
<td>Illumina Hiseq 3000</td>
<td>To identify differentially expressed RNA and pathways after EV treatment</td>
<td>III</td>
<td>Method optimized in original publications III</td>
</tr>
</tbody>
</table>

Table 7: Methods to study specific genes and proteins. Protocols are explained in detail in the original publications.

<table>
<thead>
<tr>
<th>Method</th>
<th>Purpose</th>
<th>Original publication</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Western blot</td>
<td>To analyze proteins</td>
<td>III</td>
<td>(Towbin, Staehelin and Gordon, 1979; Burnette, 1981)</td>
</tr>
<tr>
<td>Quantitative PCR</td>
<td>To quantify mRNA expression</td>
<td>II, III</td>
<td>Method optimized in original publications II and III</td>
</tr>
<tr>
<td>siRNA/plasmid transfection</td>
<td>mRNA knockdown/mutant gene expression</td>
<td>I, II, III</td>
<td>(Fire et al., 1998; Hamilton and Baulcombe, 1999)</td>
</tr>
<tr>
<td>4-MU, Mannose, and glucosamine treatments</td>
<td>To enhance or inhibit HA and / or UDP-sugar synthesis</td>
<td>I, III</td>
<td>Method optimized in original publications I and III</td>
</tr>
</tbody>
</table>
5 RESULTS

During tumor progression synthesis of HA has been shown to play a crucial role (Tammi et al., 2008). The molecular rational behind HA influencing cell behavior has been the much-studied area (Toole, 2004). But we were more interested in studying the reason behind simultaneous increase in HAS gene expression and HA synthesis in many cancers. We were also interested to know how HAS and HA were released in the EVs and whether they exert biological functions on the neighbouring cells. Our objective here was to solve these questions. This chapter summarizes the main results obtained in this thesis. Detailed data can be found in the original publications (I-III).

5.1 INFLUENCE OF HAS3 TRAFFICKING ON EV SECRETION IN MV3 MELANOMA CELLS OVEREXPRESSING GFP-HAS3

5.1.1. UDP-sugars on HAS3 trafficking

In this study, HAS3 trafficking and its residence time in the plasma membrane was investigated by controlling the availability of the UDP sugar substrates. The hypothesis was to see if subjugating the UDP sugar availability had impact on HAS3 trafficking and will that reciprocate on HA synthesis and EV secretion. To investigate this (I), we chose melanoma cells with a doxycycline inducible GFP-HAS3 (MV3-GFP HAS3).

MV3 melanoma cells stably overexpressing GFP-HAS3 (or MV3 GFP-HAS3 cells) were subjected to various treatments to investigate if these would affect the HAS3 kinetics in the cells. Cells were subjected to 4-MU, mannose, UGDHi, GFATi and GNPDAi treatments to decrease the levels of UDP-sugars that serve as the building blocks of HA. Conversely, glucosamine treatment increased the cellular concentration of UDP-GlcNAc (I, Figure 2A, B). Following the treatments, cells deprived of the UDP-sugars displayed low levels of HA secretion while glucosamine treatment increased the levels of HA secretion (Figure 2 C). UDP-GlcNAc functions as the substrate that regulates protein functions through O-GlcNAcylation. O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) are enzymes that add and remove the O-GlcNAc moiety. Cells were treated with siRNA against OGTi and an inhibitor (thiametG) for OGA. Manipulation of O-GlcNAcylation of HAS3 in MV3 GFP-HAS3 cells showed that they did not affect the UDP-sugar content but influenced the HA secretion (I, Figure 2).
All the above-mentioned treatments were further used to study the trafficking of GFP-HAS3 to the plasma membrane. A cleavable hydrophilic biotin-DTT was used to label the extracellular part of GFP-HAS3 (I, methods section), which aided in tracking of the movement of GFP-HAS3 between the plasma membrane and the Golgi. Based on this data, it was found that GFP-HAS3 was recycled back to the plasma membrane more efficiently when cells were treated with glucosamine or thiametG, while there was less recycling and more endocytosis with mannose, 4-MU and siRNA treatments against UGDH, GFAT and GNPDA (I, Figure 5B). The results showed the availabilities of UDP-GlcNAc and UDP-GlcUA influence the presence of HAS3 in the plasma membrane.

5.1.2. HAS3 trafficking and plasma membrane residence controls release of HAS3-EVs

MV3-GFP-HAS3 cells were viewed under the TIRF microscope to study the dynamics of GFP-HAS3 intracellular vesicles under the TIRF zone (100-200 nm under the plasma membrane) thereby monitoring the HAS3 recycling. The microscope was set to record the activity for 2 mins at 0.5 s intervals and the kinetics were monitored for the initial 70s. Vesicles carrying GFP-HAS3 showed an increase in recycling towards the plasma membrane when treated with glucosamine and thiamet G. The recycling of GFP-HAS3 vesicles to plasma membrane was reduced when they were treated with 4-MU, mannose and siRNAs against GFAT, GNPDA and OGT as mentioned in Table 8 (I, Figures 6, 7E). The longer the plasma membrane retention of active GFP-HAS3, the higher was the HA production accompanied by the secretion of EVs from the tips of plasma membrane protrusion (Rilla, Pasonen-Seppänen, et al., 2013). The EVs were detected budding off directly from the tips of the filopodia-like structures carrying GFP-HAS3A. The appearance of long plasma membrane protrusions is a signature organelle, which occurs as a result of high HA synthesis (Kultti et al., 2006b).

MV3-GFP HAS3 cells with overexpression of GFP-HAS3 showed longer plasma membrane protrusions and released higher amounts of EVs containing GFP-HAS3 into the surrounding type I collagen matrix during glucosamine treatment (I, Figure 8 A, B). But when treated with 4-MU, UGDHi, mannose, GFATi and GNPDAi, the matrix had a significantly lower number of GFP-HAS3 EVs. Furthermore, EV secretion decreased when O-GlcNAcylation was inhibited by OGTi, and increased due to thiametG enhancing the O-GlcNAcylation (I, Figure 8 A, B). The above results show that the substrates i.e. UDP-sugars, regulate GFP-HAS3 trafficking to
plasma membrane and its protrusions, which play a pivotal role in secretion of EVs into the surrounding environment.

Table 8. Effect of treatments on UDP-sugar level, HA secretion, EV secretion and vesicle dynamics in MV3-GFP HAS3 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>UDP-GlcNAc:UDP-GlcUA content</th>
<th>HA</th>
<th>EV secretion</th>
<th>Vesicle dynamics in TIRF zone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucosamine</td>
<td>: ↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Mannose</td>
<td>↓ : ↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>GFATi</td>
<td>↓ : ns</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>GNPDAi</td>
<td>↓ : ns</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>4MU</td>
<td>ns : ↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>UGDHi</td>
<td>↓ : ↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>OGTi</td>
<td>ns : ns</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>ThiametG</td>
<td>ns : ns</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
</tbody>
</table>

↑ - Increase; ↑↑ > 2 fold increase; ↓ Decrease; ↓↓ < 0.5 fold; ns – not significant

5.2 HA COATED EVS FROM MSCS

5.2.1 Composition and structure of EVs released from hMSCs

The human mesenchymal stem cells (hMSCs) are found to innately possess regenerative properties (Marote et al., 2016). Also, it has been shown that all types of cells including stem cells secrete EVs as a mode of communication (Fatima and Nawaz, 2015). These properties make the stem cells and the EVs secreted from them a potential target for therapeutic research. The hypothesis behind this study (II) was to visualize the impact of HA secretion and HAS expression on hMSCs and their EV secretion. We chose stem cells in this study due to their importance in therapeutics and to see if HA or HA coated EVs could be used for diagnostic purposes.

The human mesenchymal stem cells (hMSCs) were stained using a fluorescently labelled HA binding complex (fHABC) and viewed with confocal 3-dimensional (3D) imaging. The staining showed that the cells and long filopodia emerging from
the cells were coated with HA and the EVs attached to bottom of the plate were also carrying a thick coat of HA around them (II, Figure 1F, I). TEM was utilized to reveal the ultrastructure of the EVs isolated from hMSCs (II, Figure 2D). Due to fixing, the cells shrunk and collapsed the filopodia, but nonetheless CD44 immunostaining of hMSC showed that EVs were being secreted and directly budding from the plasma membrane (II, Figure 2 A-C).

NTA analysis of the isolated EVs revealed that only 3.5% of EVs were between 300-1000 nm size while 96.5% of the EVs were in a size range under 300 nm (II, Figure 2E). The HA content in the EV fraction was determined to be about 11 ng/10,000 cells. The mRNA expression levels of HASs and CD44 in the EVs mirrored the level of mRNA in parental hMSCs (II, Figure 1 B, C). The surface morphology of EVs visualized with SEM revealed their rough structure and size variability (II, Figure 3 E, F). Correlative light and electron microscopy (CLEM) illustrated that the shedding of EVs was not only limited to blebbing from filopodia or plasma membranes but the retracting filopodia/fibers formed “footprints”. Images also showed a partial colocalization of CD44 and HA in both cells and EVs and their release in EVs from the plasma membrane protrusions (II, Figure 4). Based on these results we were able to conclude that hMSCs were producing high amounts of HA and the EVs secreted from hMSCs also carried HA. The EVs released from the long plasma membrane protrusions were similar to the EVs released from MV3-GFP HAS3 cells from study I.

5.2.2 Effect of HAS enzymes on hMSCs and its EVs

The secretion of HA, budding of EVs, the architecture of filopodia and expression levels of HASs were altered when MSCs were treated with lipopolysaccharide (LPS). MSCs grown in 3D cultures (basement membrane extract gel) acquired a spindle shaped morphology with the majority of the protrusions being CD44 positive. There was a higher level of HA staining around the cells as compared to control and the EVs of varying sizes were seen budding from the protrusions and their tips (II, Figure 5 A-E). While LPS treatment did not have a significant effect on the secretion of the EVs (II, Figure 5 I), it increased the HA secretion and content in the cells and EVs (II, Figure 5 H, J). mRNA levels of HAS1-3 increased across all concentrations of LPS treatment (II, Figure 5 K-M). The cells were treated with HAS siRNAs and 4-MU to observe if knocking-down of HAS and HA synthesis had any effect on MSCs. The HA synthesis in MSCs was not changed when HAS3 was inhibited whereas it decreased by 47 % when HAS2 was inhibited and a 44 % decrease was observed when HAS2 and 3 were simultaneously inhibited. HAS
knockdown did not significantly affect the HA content in EVs, but it mirrored the HA synthesis in the cells (II, Figure 6 C, K). 4-MU treatment decreased HA secretion in the cells leading to similar changes in the HA content of the EVs. The mRNA expression levels of HAS1 and 2 decreased significantly while the HAS3 level increased although the change was not significant (II, Figure 8 E, F, H-J). HAS knock-down with siRNA and 4-MU did not have any effect on the EV secretion levels (II, Figure 6 J, 8 G). CLEM images revealed a decrease in cell-associated HA around the filopodia with HAS2 silencing (II, Figure 7). However, a decrease in CD44 immunostaining in cell surface protrusions and EVs, and a decrease in the amount of cell associated HA was observed only with 4-MU treatment (II, Figure 8 A-D). The results suggest that inhibition of HA synthesis activity by HAS silencing and 4-MU treatment do not affect the EV secretion rate, but they have a slight effect on the HA content of EVs, reflecting the changes in the HA synthesis rate.

5.3 MELANOMA CELLS DERIVED HAS3-EVS AND THEIR FUNCTIONS

5.3.1. GFP-HAS3 EVs: Characterization and functions exerted on target cells

Our previous studies (I, II) showed that post-translational regulation of HAS using UDP sugar substrates had a complete or partial effect on HAS3 trafficking, which simultaneously controlled HA secretion and EV release. Based on these we investigated if HAS- and HA- coated EVs were able to induce any biological functions by signaling mechanisms that could impart tumorigenic properties on the target cells (i.e. the cells that interact with the EVs). Also, to investigate the cellular mechanisms involved in the shedding of EVs from the donor cells (i.e. the cells that secrete the EVs).

For this study (III) MV3-GFP HAS3 cells were used, as these cells enabled us to control GFP-HAS3 expression through its doxycycline induction system. We contemplated that by using these cells it would be straightforward to learn the biological functions stimulated by HAS3-EVs compared to control EVs. HaCaT cells were used as target cells (III) due to their low endogenous levels of HA.

Human MV3 melanoma cells, expressing doxycycline (DOX) inducible GFP-HAS3 were grown in type I collagen matrix to entrap and visualize the cells and the EVs by confocal microscopy. When induced by DOX, the cells, had more GFP-HAS3 positive EVs (HAS3-EVs) and filopodia compared to uninduced cells. EVs from uninduced cells were called MV3-EVs. The EVs secreted from induced cells had a thick coat of HA around them (III, Figure 1A-H). Western blotting showed that both
HAS3- and MV3-EVs were positive for the HA receptor CD44 and EV markers CD81, CD63 and actin (III, Figure 1I). The particle concentration of HAS3-EVs group was 75% higher than that in the MV3-EVs group but the size distribution between the groups was similar (III, Figure 1K, L).

The results showed that HAS3-EVs utilize the CD44 receptor as one of their modes to bind to the target cells and HA synthesis of the HaCaT cells increases when they are treated with HAS3-EVs. HAS3 and MV3-EVs regulated the biological functions of the recipient cells. The cell proliferation rate of HAS3-EV-treated HaCaT and WM115 cells increased significantly by day 4 as compared to cells treated with MV3-EVs. A similar trend was observed with the invasion rate of the recipient cells when treated with the EVs (III, Figure 2). These results indicate that the unidentified cargo in HAS3-EVs was inducing the tumorigenic properties of recipient cells.

5.3.2. Comprehensive profiling of HAS3-EVs treated recipient cells for identification of key players

HaCaT cells treated with HAS3-EVs were lysed and subjected to an antibody array consisting of cell cycle related proteins. The array results showed an elevation in expression of proteins involved in cell division and transcription such as cyclin E, E2F1, E2F2, Ki97, CDK1 and cullin-3 (III, Figure 3A). Label-free quantitative analysis on the whole cell proteome of HaCaT cells treated with HAS3-EVs and MV3-EVs was performed. Claspin was identified as one of the topmost differentially regulated proteins (1.95-fold change) in HAS3-EVs treated cells. In comparison, the claspin expression level in cells treated with MV3-EVs showed a difference of 0.86-fold change (III, Suppl. Table1). Claspin, a nuclear protein has a role both in cellular homeostasis and DNA replication. A decrease or increase in cell proliferation rate directly corresponds with claspin protein levels (Yang et al., 2016; Azenha et al., 2017). Pathway analysis with IPA software indicated that the pathways belonging to cell proliferation, cytoskeletal rearrangements and epithelial adherens junction remodeling were elevated in cells treated with HAS3-EVs (III, Figure 3B). High throughput RNA sequencing detected an increased expression level of GLI1 and PTCH1 in cells treated with HAS3-EVs. These genes belong to the hedgehog signaling pathway whose downstream target is c-Myc as well as many other genes (III, Figure 3C). Western blotting analysis displayed an enhanced expression of c-Myc in HAS3-EVs treatment, when compared to HaCaT cells treated with MV3-EVs (III, Figure 4A-D, Suppl. Figure 1A). These targets together
point to a plausible association of the hedgehog signaling pathway with c-Myc and claspin, which could lead to activation of unregulated cell proliferation.

5.4 HAS3-EVS REGULATION OF CELL PROLIFERATION DEPENDS ON IHH MITOGEN MEDIATED MYC AND CLASPIN EXPRESSION

5.4.1. HAS3-EVs stimulate HH signaling and thereby upregulation of c-Myc and claspin

The protein levels of Gli-1 and -2, c-Myc and claspin in HaCaT and WM115 cells treated with HAS3-EVs were significantly upregulated while MV3-EVs treatment did not evoke any major upregulation (III, Figure 4, 5). Silencing of c-Myc and claspin with siRNAs decreased their expression level whereas adding HAS3-EVs achieved a partial rescue of the protein expression (III, Figure 4 A-D, F-G). Knocking-down of c-Myc decreased the expression of claspin while claspin knockdown did not affect c-Myc expression. This implied that claspin expression depends on its upstream c-Myc activation. While this claspin dependency on c-Myc is downstream of the HH pathway activation, inhibiting Gli-1 and -2 with the GANT58 inhibitor decreased the expression levels of Gli-1 and -2, c-Myc and claspin. A point mutation T58A in c-Myc constitutively activates the oncogenic functions while lowering its apoptotic ability (Conzen et al., 2000).

Cells were transfected with c-Myc T58A mutant plasmid to see if c-Myc could overcome Gli inhibition. The mutant plasmid partially rescued the expressions of certain proteins (c-Myc, claspin, Gli and slug) when the cells were treated with HAS3-EVs (III, Figure 5 A-F). The proliferation rate of HaCaT and WM115 cells decreased significantly 48h after c-Myc and claspin siRNA transfection and HAS3-EVs treatment did not aid in the increase of proliferation (III, Figure 4 E, and H). Gli inhibitor treatment caused a similar decrease in the proliferation of the cells and HAS3 EVs along with c-Myc T58A treatment increased the proliferation rate on a par to the control (III, Figure 5 G, H). The results suggest that HAS3-EVs stimulate the HH signaling pathway leading to increased expression of genes related to proliferation by c-Myc activation.

5.4.2. HAS3-EVs carry IHH to stimulate the HH signaling pathway

All the above results exhibited that HAS3-EVs triggered HH signaling pathway in the target cells. The mitogen carried by the EVs that caused this upregulation in the target cells still needed to be analysed. The presence of mitogens in MV3-and
HAS3-EVs that are responsible for triggering the HH pathway were analyzed using western blotting. Among the different mitogens, IHH showed a remarkably higher expression in HAS3-EVs, when compared to MV3-EVs (III, Figure 6C). In HAS3-EVs, while EGF expression was detected, IHH expression was stronger, implying that it is the main player in the components of the HAS3-EV (III, Figure 6C). Induction of GFP-HAS3 in MV3 cells resulted in an upregulation of HH genes GLI1, PTCH1, SMO, GLI1 and -2, while mannose treatment decreased their expression (III, Figure 6A). Immunostaining of human melanoma sections with claspin antibody revealed that the stromal areas were negative while epithelial cells and melanocytes exhibited positive claspin staining. Claspin staining intensity was moderate or weak in dysplastic melanocytes and in situ melanoma and weak or negative in invasive melanomas. This staining pattern correlated with HA staining during melanoma progression (III, Figure 7 D, E). These results indicate that IHH triggers the HH-c-Myc-claspin signaling axis that induces the tumorigenic properties of the recipient cells.

5.5. HA AND HH PATHWAYS HAVE A POSITIVE FEEDBACK REGULATION

In MV3-GFP-HAS3 cells, mannose treatment reduced the expression level of some of the HH genes and GANT58 treatment reduced the secretion of HA (III, Figure 6 B). This indicates that there is a positive feedback regulation mechanism between HA synthesis and HH signaling. The HA secretion of HaCaT cells did not show any increase when the cells were treated with EVs collected from parental MV3 cells that had been transfected with the GFP-ΔHAS3 plasmid (GFP-ΔHAS3 EVs) (III, Suppl. Figure 2 D). The same EVs had IHH protein levels similar to control while wild type GFP-HAS3 EVs possessed increased IHH levels. Silencing HAS2 and 3 simultaneously in MV3 cells showed a reduction in the IHH expression levels (III, Suppl. Figure 2 E). HA secretion of HaCaT cells was decreased after treatment with EVs collected from MV3 GFP-HAS3 cells after IHH knockdown (III, Suppl. Figure 2 F). These results suggest that HA signaling is responsible for IHH secretion in EVs and these EVs influence the signaling mechanisms in recipient cells.
6 DISCUSSION AND CONCLUSION

6.1 EVS GOVERNED BY MESENCHYMAL STEM CELLS

Multipotent mesenchymal stem cells (MSC) have been a topic of active research during recent years due to their therapeutic and regenerative potential in diseases such as cancer, Alzheimer’s disease, myocardial infarction and osteoarthritis. The EVs released from MSCs mimic the characteristics of their parental cells and this general property of the EVs has therapeutic potential. SCID mice with cisplatin-induced acute kidney injury showed an enhanced survival rate when treated with MSC-derived EVs. This study suggested that the improved survival of the mice was due to the EVs which conferred a protection mechanism on the cells by upregulating anti-apoptotic genes (Timmers et al., 2008). The EVs interaction and delivery to recipient cells is driven by specific surface receptors and ligands carried by the EVs which makes them non-invasive vehicles for regenerative medicines.

The microenvironment surrounding the stem cells contains various cellular and extracellular factors that support the formation of the stem cell niche (Li and Xie, 2005; Jha et al., 2011). HA, which mediates the transition of the progenitor cells to mesenchymal cells, is also known to play an important role in the differentiation of hESC (embryonic stem cells) into the HSCs lineage (hematopoietic stem cell) (Schraufstatter et al., 2010; Shukla et al., 2010; Solis et al., 2012). It has been shown that stem cell niches are rich in HA, furthermore, one of the markers for stem cells is CD44, a receptor that interacts with HA (Zöller, 2015). As shown previously in immortalized cells (Rilla, Pasonen-Seppänen, et al., 2013; Rilla et al., 2014), in this project, it was demonstrated that also primary MSCs exhibit filopodia and secrete EVs coated with HA. These EVs had a variable size range, a high concentration of HA and expression of CD44 (II). CD44 aids in the homing of MSCs in the bone marrow through interactions with HA, enabling the cells to maintain their undifferentiated state. The results of this study suggest that CD44 is also a potential biomarker for MSC derived EVs.

The CD44-HA interaction triggers a signaling cascade, activating ERK and MEKK1, which induces differentiation, proliferation and EMT in ESCs (Kothapalli et al., 2008; Craig et al., 2010; Hatano et al., 2011). The EVs’ mode of communication with the ECM and recipient cells could be mediated through the interaction of CD44 with HA. It has been also shown that CD44 positive EVs are secreted from primary mesothelial cells when they are induced to undergo EMT through injury or by
adding EGF (Koistinen et al., 2017). MSCs produce high levels of HA endogenously, and furthermore inhibition of HA synthesis by 4-MU significantly decreased the formation of HA-coated filopodia and lowered the expression of HAS1 and 2 (II). 4-MU treatment of human embryonic stem cells decreased HA secretion, leading to poor mesodermal differentiation (Schraufstatter et al., 2010). Surprisingly, HAS3 was not affected by 4-MU which could have contributed to the failure to prevent EV secretion (II). During embryogenesis, HAS2 has been shown to be a significant source of HA (Camenisch et al., 2000a). The HA content of the EVs during all the treatments reflected the activity in the parental cells, indicating that the changes in the biological properties of the EVs could be used for disease monitoring and diagnostic purposes (II). Previously, EV shedding has been linked to HAS activity and the availability of HAS substrates is the key regulator of the extent of EV secretion (Rilla, Pasonen-Seppänen, et al., 2013; Rilla et al., 2014). Stem cell research is an important area because of the potential of these cells to differentiate into any cell type. HA plays a significant role in stem cell functions and it also interacts with numerous ECM components (Solis et al., 2012). Many recent studies have investigated the healing properties of MSC-EVs in myocardial ischemia, chronic heart failure and acute kidney injury (Bruno et al., 2009; Lai et al., 2010; Kervadec et al., 2016).

In numerous animal models, MSC-derived EVs have exhibited promising therapeutic effects by participating in cutaneous wound healing via Wnt4 signaling (Zhang et al., 2015). EVs isolated from hMSCs can be used for therapeutic purposes as they are unable to form tumors directly and have a lower susceptibility to trigger adaptive and innate immunity in patients (Rani et al., 2015). Pascucci et al. have shown that paclitaxel treated hMSC-EVs were able to inhibit cell proliferation and they induced a 50% reduction in human pancreatic adenocarcinoma (Pascucci et al., 2014). EVs originating from stem cells carrying HA could be potential mediators in regeneration and tissue healing thereby increasing the therapeutic efficiency. MSC derived EVs can evoke effects on neighboring cells or communicate via paracrine or autocrine signals through CD44/HA carried by the EVs. This study (publication II) presents a detailed picture of the structure of MSC-EVs, as well as their content, therapeutic potential and the influence that HA and HASs exert on their secretion. This study revealed that it would be advantageous to clarify the trafficking of HASs and their impact on EV secretion.
6.2 EFFECT OF HAS3 RECYCLING IN THE SECRETION OF EVS

Retention of HAS enzymes in the plasma membrane maintains the synthesis of HA on the surface of the cells, leading to the secretion of HA chains directly into the extracellular space. It must be noted that not every HAS3 protein reaching the plasma membrane will initiate HA synthesis. Those HAS3 proteins which do not stay in the plasma membrane are directed either towards lysosomal degradation or recycled back to the plasma membrane (I). Proteins that go through recycling or endocytosis could actually be released in EVs (Vidal et al., 1997; Fang et al., 2007; Muntasell et al., 2007). There is a direct association between HA synthesis and EV secretion in cells with increased HAS expression (Rilla et al., 2013). One could speculate that cells with increased HAS expression and thereby an elevated HA content utilizes HA-coated EVs as one of the cell-to-cell communication tools to spread HA-mediated molecular machineries and implications. During HA secretion, HAS3 is present on the plasma membrane, especially on the filopodial membranes, from where a significant proportion of the EVs bud and carry HAS3 on their membranes. Co-passengers of HAS3 in EVs released from the cells having active HA synthesis were found to be CD44 and actin (Rilla et al., 2013). Other examples of proteins that follow a similar intracellular pathway as HAS3 include matrix metalloproteinases (Hakulinen et al., 2008), EGFR (Adamczyk et al., 2011), cytokines (Konadu et al., 2015) and integrins (Fedele et al., 2015b). Even though some of the HAS3 reaching the plasma membrane stay longer to allow the growing HA chains to mature, the average half-life of HAS3 in the plasma membrane is ~5-6 mins (Deen et al., 2014). This shows that there is an active flux of HAS3 in the plasma membrane and its vicinity, and only these enzymes that initiate HA synthesis stay in the membrane. Hence, an active HAS3 enzyme is required at the plasma membrane for HA synthesis and thus understanding the retention time and recycling of HAS3 is an integral part of the process. The concentrations of UDP-sugars in the cell are influenced by the amount of glucose which is present; this means that cellular availability of glucose regulates HA synthesis and HAS3 transport (Rilla, Oikari, et al., 2013). In addition to being part of HA synthesis, UDP-GlcNAc is involved in O-GlcNAcylation of proteins to control many vital cellular functions. Levels of UDP-GlcNAc in the cell reflect the metabolic status and level of glucose in the cells (Buse, 2006).

The absence of both UDP-GlcUA and UDP-GlcNAc substrates of HAS3 downregulates HA synthesis and HAS3 transport to the plasma membrane (I). Itano et al. have shown that the Km values of HAS3 correlated with the substrate
levels, confirming that altering the substrate levels could impact on the activity of HAS3 (Itano et al., 1999). Biotinylation of HAS3 and TIRF analysis demonstrated that UDP-sugars and O-GlcNAcylation control the HAS3 retention time on the plasma membrane. Treatment with glucosamine and thiamet G enhanced the transfer of HAS3 to the plasma membrane and this led to an increased shedding of HA coated and HAS3 positive EVs (I). Our current (I) and previous works (Rilla, Pasonen-Seppänen, et al., 2013) suggest that the HA chains remain attached to HAS3 on the EV membranes. An increase in UDP-GlcNAc and O-GlcNAcylation can circumvent the lysosomal degradation of HAS3. This could be an added advantage as it increases the availability of HAS3 on the plasma membrane and its release in EVs. Since it is known that extracellular HAS3 accumulation takes 24-48h (Rilla, Pasonen-Seppänen, et al., 2013; Deen et al., 2014), it is difficult to quantify the proportion of HAS3 shed in EVs as compared to those endocytosed. Clarifying the functions exerted by EVs carrying HA and HAS3 could increase our knowledge about the communication between cancer cells and neighboring cells and tissues. This study (I) provides further insights into the intracellular movement of HAS3 and the shedding of HAS3 extracellularly in EVs. Though the exact role of HA induced EVs will require further investigation, it is possible that the EVs could bind to the target cells using CD44 and trigger downstream signaling mechanisms. These results suggest that the cargo of those EVs carrying HA is unique as compared to HA-negative EVs. Furthermore, an awareness of the level of the impact that glucose metabolites have on HA and EV secretion could be vital in understanding the progression of tumors-like melanoma.

6.3 HAS3-EVs TRIGGER A SIGNALING PATHWAY IN RECIPIENT CELLS

EVs are a lipid bilayer enclosed particles that carry cellular and genetic information of the donor cells and which are transported to the neighboring cells as a mode of communication. In this thesis work (I, II), we studied the occurrence of EVs carrying HA and their mode of release from the donor cells. Furthermore, we investigated the signaling mechanism triggered by these EVs in their target cells. Communication between cells forms the basis for the maintenance of homeostasis in the cellular environment and cancer cells use EVs as one of their communication modes to induce aberrant changes in the signaling pathways and to disturb the biological functions of normal cells (Naito et al., 2017). For example, mammary epithelial cells attained anchorage independent growth when treated with breast cancer cell-derived EVs (Antonyak et al., 2011). Similarly the invasive capacity of normal hepatocytes increased when the cells were exposed to EVs from
hepatocellular carcinoma cells (He et al., 2015). Our current study demonstrated that HAS3-EVs from metastatic melanoma cells induced an unregulated proliferation and EMT (one of the hallmarks of cancer) in keratinocytes (i.e. HaCaT) and also in the less aggressive melanoma cells (i.e. WM115). The HAS3-EVs binding to the recipient cells was partially inhibited when the target cells were treated with short HA oligosaccharides that block HA binding to CD44 receptors (III). This is in line with our previous results (II) showing that both HA and CD44 are carried by EVs. Previous studies have shown that SHH is transported in EVs released from the apical microvilli during embryonic development of the mouse ventral node (Briscoe and Thérond, 2013). HAS3-EVs released from the melanoma cells also carry the IHH mitogen which triggered the HH pathway in the target cells. While most of the studies have shown the SHH mitogen to be a participant in tumor progression (Mas et al., 2007; O’Reilly et al., 2013; Faião-Flores et al., 2017), the finding that IHH was one of the prime mitogens in HAS3-EVs was unexpected. IHH has been reported to contribute to the proliferation and self-renewal in some cancers like lung cancer (Zhang and Hu, 2018), colorectal cancer (Pelillo et al., 2015) and T-cell acute lymphoblastic leukemia (Dagklis et al., 2016). The aberrant activation of the HH pathway in the cancer cells can also occur through overexpression of the HH ligands. There are findings in the literature indicating that HH activation triggers the expression of various proliferative genes leading to an increased rate of proliferation in tumor cells (Berman et al., 2003; Evangelista, Tian and De Sauvage, 2006). IHH in the EVs trigger the HH signaling pathway in the target cells and this pathway is the direct upstream regulator of proto-oncogene c-Myc (III).

Upregulated proliferation in cancer cells can be induced by various proteins and transcription factors, like c-Myc and E2F family of transcription factors (Leung et al., 2008) that exert a direct control over CDKs, cyclins and various growth promoting genes (Hallmann, 2009; Narasimha et al., 2014). Increased levels of c-Myc enhance the interactions with the E2F promoter, leading to an increase in the expression of E2F regulated genes (Fernandez et al., 2003). The interaction of HAS3-EVs with target cells shows a remarkable increase in c-Myc, E2F1 and E2F2 proteins, resulting in increased expression levels of claspin. Claspin protein is a downstream regulator of c-Myc, acting as a checkpoint kinase monitoring replication stress. Recently it was observed that the acid patch sequence in the C terminal of claspin is responsible for non-checkpoint functions (Yang et al., 2016). The expression of claspin is regulated by E2F, an S phase dependent transcription factor. A deregulated E2F pathway is another contributor to the increased claspin levels (Smits et al., 2018). Inhibiting GLI1/2 expression decreased both c-Myc and claspin
downstream expression. c-Myc expression was partially rescued by the transient transfection of c-Myc T58A mutant even under GLI inhibition, indicating that in this study, the HH pathway had been the upstream regulator of c-Myc. Upregulation of the HH pathway in rat prostate adenocarcinoma through overexpression of GLI1 was able to induce cancer cell metastasis through a stimulation of EMT genes (Karhadkar et al., 2004). Increased proliferation and the expression of EMT markers were seen in recipient cells after treatment with HAS3-EVs. However, cellular proliferation and expression of EMT markers were decreased, when claspin, c-Myc and GLI1/2 were downregulated. Recent studies have shown that the promoter activity of chk1 and claspin (i.e. proteins expressed at the replication fork during stress) are upregulated when E2F transcription factors are activated by c-Myc (Bertoli et al., 2016; Smits et al., 2018). While E2F and c-Myc were the key players in this study, cells could use also claspin to increase the proliferation rate. This could perhaps be one of the tactics exploited by cells to elude the replication stress occurring during tumorigenicity. The increases in EMT and proliferation, which are evident due to an enriched G1/S phase, are observed in recipient cells treated with HAS3-EV since this triggers the IHH-c-Myc-claspin signaling axis (III).

6.4 A NOVEL FEEDBACK REGULATION BETWEEN HH AND HA

The hedgehog signaling pathway is normally acting as a developmental morphogen, but it also modulates the invasiveness and metastasis of cells when they have been aberrantly activated (Karhadkar et al., 2004). The dual lipidation of HH proteins by cholesterol and palmitic acid is the main reason for their retention on the plasma membrane. When they are located on the plasma membrane, the HH proteins can interact with distant cells through multimers, lipoproteins and EVs (Briscoe and Thérond, 2013). After reaching the plasma membrane, HAS proteins are activated to synthesize HA on the inner surface of the plasma membrane and to extrude the HA chain into the extracellular space (Prehm, 1984). The elevated levels of IHH found in the HAS3-EV cargo are interesting as this points toward a relationship between HH and HA. Additionally, silencing of HAS2 and HAS3 in melanoma cells decreased the levels of HH pathway genes and sorting of IHH into the EVs. On the other hand, inhibition of GLI1/2 decreased HA secretion in the melanoma cells (III). Cancer promoting factors, such as Akt and ZEB1 have been shown to exert a positive feedback with HA (Liu and Cheng, 2017; Maurer et al., 2017). However, in one study, these factors have shown to be able to regulate the HAS2 gene by GLI1 and 2 that bind to the HAS2 promoter (Liu et al., 2013). This
relationship between HH and HA can be observed when EVs collected from melanoma cells with IHH silencing failed to induce HA secretion in the target cells (III). EVs containing a truncated version of HAS3 that has only 86 amino acids displayed a decrease of IHH in the EVs and in HA secretion of the target cells, emphasizing that the catalytic activity of HAS3 is required for all above observed effects. Our results demonstrate a positive feedback regulation between HH and HA in melanoma cells and the EVs secreted from them can trigger unregulated proliferation and EMT in their target cells (III). The similarity that we detected here is that both HH and HA are active on the plasma membrane, both have functions during embryogenesis and furthermore the unregulated pathway activation of HH and HA leads to cancer (Evangelista, Tian and De Sauvage, 2006; Tammi et al., 2011). In many types of cancer, the pathological effects have been shown to be exerted by the HH pathway (Kim et al., 2018; Yang et al., 2018) and HA (Siiskonen et al., 2013; Kultti et al., 2014b) individually, but this study is the first to show their synergistic effect, particularly in melanoma. This finding can help us to understand the ways by which cancer cells acquire resistance to certain drugs and enhance their relapse strategies.

6.5 HA CARRYING EVS AS BIOMARKERS

Cancers in general are receptive to chemotherapy-based treatments, but they are able to develop resistance to the treatments over time due to metabolic changes and random mutations that occur in their DNA and proteins (Housman et al., 2014). A mutation in the BRAF protein at Val600 (valine position 600) that leads to constitutive activation of MAPK pathway occurs in 40-50% of melanoma patients (Colombino et al., 2012). Usually for patients with the BRAF mutant, treatment using BRAF inhibitors (BRAF-i) is effective. However, in some cases, combinatorial immunotherapeutic drugs like dabrafenib, cobimetinib, trametinib and vemurafenib that target both BRAF and MEK are required for improved effects in patients having advanced metastatic melanoma. Ipilimumab (anti-CTLA4 antibody), oncolytic virus talimogene laherparepvec (TVEC) and inhibitors of PD-1 protein such as pembrolizumab are currently drugs approved in the metastatic stages of melanoma. Oncolytic viruses and checkpoint inhibitors are an additional category of immunotherapeutic drugs that have shown enhanced efficacy (Hogan, Levesque and Cheng, 2018). While there have been developments and the discovery of drugs, nearly 50% of the patients do not respond to the immunotherapy treatments. The survival rate for the patients not experiencing any cancer progression ranges between 2.9 – 11.5 months (Franklin et al., 2017; Hogan,
Levesque and Cheng, 2018). At this point, the use of EVs as biomarkers could aid in screening disease progression, targeting signaling pathways, targeting explicit mutations and above all in monitoring patient survival and the response to the treatments.

Peripheral blood samples from melanoma patients undergoing anti-CTLA4 immunotherapy expressed a gene signature of MYC, CDK2 and 13 others differentially expressed genes. During the therapy, these gene signatures were prognostic and predictive and showed an overall survival rate of 1 year in melanoma patients who were treatment naïve for tremelimumab (Friedlander et al., 2017). EVs were found to be more enriched in the lymph and the EVs bear tumor specific markers such as integrins, tetraspanin, Rab-GTPases and ICAM-1 (Broggi et al., 2019). EVs derived from the melanoma patients’ lymph carried the V600E BRAF mutation and it was found that in patients, this mutation was associated with relapse of cancer (García-Silva et al., 2019). These studies demonstrate that melanoma derived EVs detected from lymph could be used for biomarker analysis and are a promising tool in predicting the response to therapy. The EVs released from melanoma cells with high levels of HA secretion could be a possible tool for conducting a biomarker study i.e. the HA and IHH content of the cargo could be manipulated using the positive feedback regulation between HH and HA. This thesis work demonstrates for the first time that melanoma cells overexpressing HAS3 produce EVs enriched with the IHH mitogen. These EVs are potential predictors for monitoring melanoma progression and the response to therapy, especially, during the initial stages of melanoma.
Figure 5: Summary representing each of the studies included in this thesis. A) MV3 GFP-HAS3 cells when treated with doxycycline overexpress GFP-HAS3 and release high amounts of HAS3- and HA-coated EVs. Glucosamine treatment further increases the release of such EVs. Treatments with mannose and 4-MU decreases HAS3- and HA-coated EV secretion. During the treatments, along with EV secretion, an increase and decrease of HA synthesis and composition of EVs are also affected (Study I, III). B) Secretion of EVs from hMSCs and their composition are shown (Study II). C) EVs released from MV3 GFP-HAS3 cells interact with target cells (HaCaT and WM115). Once they interact, the mechanistic and functional impact on these cells can be seen. A cross sectional view of the HAS3 EVs with its cargo is shown for representation (Study III).
6.6 CONCLUSIONS AND FUTURE DIRECTIONS

This thesis work sheds light on the prospects of exploiting HA-rich EVs secreted from melanoma and mesenchymal stem cells, as well as clarifying their shedding mechanisms from the donor cells and their interaction with their recipient cells.

The finding that EVs from hMSCs carry both CD44 and a HA coat around them is important and opens new horizons for understanding how stem cells communicate with their environment as well as the properties that allow them to regenerate. Even though the decrease in the levels of UDP-sugars or HASs had no impact on the EV secretion levels, the decreased synthesis of HA in the cells was reflected in the lower HA content in the EVs. Melanoma cells overexpressing HAS3 secreted increased levels of EVs into the extracellular matrix during glucosamine treatment or when O-GlcNAcylation was induced. The reduction in EV secretion because of the decline in the amounts of UDP-sugars revealed the strong dependence of HAS3-EV shedding on the availability of substrates. The HAS3-EVs were able to trigger the IHH-c-Myc-claspin signaling axis in recipient cells, leading to enhanced proliferation and EMT. Staining of tissue sections from different stages of melanoma with claspin displayed an expression pattern that correlated with the staining intensity of HA. The HAS3-EVs were able to induce tumorigenic properties in the recipient cells, indicating that EVs released from cancer cells carry factors that induce tumorigenic properties in normal or at least less malignant cells. The melanoma cells expressed a positive feedback regulation between hedgehog signaling pathways and HA that governed the packing of IHH in the EVs. This is an important finding, suggesting that IHH carried in melanoma derived EVs could be a possible biomarker as it is coupled with HA. The HA content varies in different stages of melanoma and along the course of its progression, with the highest content seen in in situ melanoma tissues (Siiskonen et al., 2013). It would be enlightening to determine if IHH is released in EVs and whether a possible feedback regulation between HA and HH pathways would be important during the early stages of melanomagenesis.

In the future, it would be interesting to study in more detail the HA-dependent mechanism that is responsible for the packaging and release of IHH in the EVs and to determine if IHH is able to activate in parallel with other pathways in the recipient cells. A focus of using IHH and HA combined for therapeutic purpose would serve towards developing drugs for combatting cancer. A better
understanding of the MSC derived HA-EVs and their impact on target cells is needed to open novel ways for developing therapeutic and regenerative medicines. Research into EVs is a promising and rapidly expanding area which will lead to a deeper understanding of human physiology and the mechanisms behind important diseases such as cancer. However, many questions such as the specificity of each EV subset, their isolation methods and their specific functional roles remain to be addressed. Exploring the options of using EVs for the delivery of therapeutic molecules could be a promising approach as they have the capacity to carry and transfer miRNAs, mRNA, proteins and drugs to selective targets. Overall, improving our understanding of the biology of EVs may open novel applications for cancer therapies and diagnostics and represent a milestone on the road towards personalized medicine.


Cai, J. et al. (2013) ‘Extracellular vesicle-mediated transfer of donor genomic DNA to recipient cells is a novel mechanism for genetic influence between cells’,


Hoshino, D. et al. (2013) ‘Exosome secretion is enhanced by invadopodia and


Jha, A. K. et al. (2011) ‘Controlling the adhesion and differentiation of


54. doi: 10.1074/jbc.M110.127050.


Li, L. et al. (2015) ‘Transforming growth factor-β1 induces EMT by the transactivation of epidermal growth factor signaling through HA/CD44 in lung and breast cancer cells’, International Journal of Molecular Medicine, 36, pp. 113–122. doi:


van Muijen, G. N. P. et al. (1991) ‘Establishment and characterization of a human melanoma cell line (MV3) which is highly metastatic in nude mice’, *International


Prehm, P. (1984) ‘Hyaluronate is synthesized at plasma membranes.’, The
Biochemical journal, 220(2), pp. 597–600.


Vyas, N. et al. (2014) ‘Vertebrate Hedgehog is secreted on two types of extracellular vesicles with different signaling properties’, Scientific Reports, 4, p. 7357. doi: 10.1038/srep07357.


Wu, S. et al. (2013) ‘Microvesicles Derived from Human Umbilical Cord Wharton’s Jelly Mesenchymal Stem Cells Attenuate Bladder Tumor Cell Growth In


This thesis aimed at investigating the regulation of hyaluronan coated extracellular vesicles and their interaction with recipient cells. The results obtained show the prospects of exploiting HA-rich EVs secreted from melanoma and mesenchymal stem cells, as well as understanding their interaction with their recipient cells. In this thesis a more profound understanding of the functional relevance of HA synthesis and its effect on the secretion of EVs has been uncovered.