The thesis showed that UVB, the most important risk factor of skin melanoma, induces the expression of genes involved in melanomagenesis in hyaluronan-dependent way. While increased hyaluronan synthesis reduced growth of metastatic melanoma cells. Factors secreted by melanoma cells activated fibroblasts to produce hyaluronan that modulates the microenvironment suitable for tumor growth. The results help to understand the role of hyaluronan in melanomagenesis and its potential as a therapeutic target.
Hyaluronan and its role in melanomagenesis
PIIA TAKABE

Hyaluronan and its role in melanomagenesis

From melanocytes to metastatic melanoma

To be presented by permission of the Faculty of Health Sciences, University of Eastern Finland for public examination in lecture hall SN200, Kuopio, on Saturday, August 18th 2018, at 12 noon

Publications of the University of Eastern Finland
Dissertations in Health Sciences
Number 475

Institute of Biomedicine, School of Medicine, Faculty of Health Sciences,
University of Eastern Finland
Kuopio
2018
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Hyaluronan is a high molecular weight glycosaminoglycan, which is produced by hyaluronan synthases (HAS1–3). During its synthesis, it is protruded to the extracellular space. Hyaluronan can be bound to the synthase itself or to plasma membrane receptors, such as CD44, or it can be free in the extracellular space. High molecular weight hyaluronan is degraded to small oligosaccharides by hyaluronidase enzymes (HYAL). In a homeostatic stage, hyaluronan is expressed mainly as a high molecular weight polymer, while during situations like inflammation it is degraded to smaller, biologically active oligosaccharides. These molecules can foster inflammatory signaling and cytokine and chemokine production. Increased hyaluronan expression from cancer cells or cancer stromal cells, correlates with poor patient outcome in many cancers, such as breast cancer. But in melanoma, the situation is not as clear. Recent studies from patient samples revealed that benign nevi and melanoma in situ express substantial hyaluronan in the melanocytic cells but in invasive melanoma, melanoma cells are almost negative for hyaluronan. The objective of this doctoral thesis was to reveal the effect of hyaluronan in melanomagenesis. The specific aims were to study the influence of hyaluronan in the transformation of primary melanocytes to dysplastic melanocytic cells, to investigate the impact of increased HAS3 expression and pericellular hyaluronan in metastatic melanoma cells, and to determine the potency of melanoma cell’s secreted factors for stromal fibroblasts activation.

The results showed that primary melanocytes express a thick pericellular hyaluronan coat and UVB-exposure together with hyaluronidase induces strong inflammatory cytokine and chemokine expression of IL-6, IL-8, CXCL-1 and CXCL-10. When hyaluronan synthesis is increased in metastatic melanoma cells by overexpressing HAS3, cells’ proliferation was reduced due to decreased phosphorylation of signaling molecules leading to cell division (ERK, p38). Also the cells migrated less due to increased pericellular hyaluronan and showed a lower amount of focal adhesions. Melanoma cells secreted factors that activated PDGFR-mediated AKT phosphorylation, increased HAS2 expression and hyaluronan synthesis. Inhibiting PDGFR or AKT-signaling could prevent expression of HAS2 and hyaluronan synthesis.

In conclusion, this thesis provides novel data that hyaluronan plays an important role in the early changes of melanocytes towards dysplastic cells as well as in the tumor microenvironment in metastatic melanoma. In addition, targeting drug research into hyaluronan metabolism in metastatic melanoma may potentially reduce its aggressiveness and further spreading.
Takabe, Piia
Hyaluronaanin rooli melanoomageneesissä, terveistä melanosyyteistä metastaattiseen melanoomaan
Itä-Suomen yliopisto, terveystieteiden tiedekunta
Publications of the University of Eastern Finland. Dissertations in Health Sciences Numero 475. 2018. 125 s.

ISSN (print): 1798-5706
ISSN (pdf): 1798-5714
ISSN-L: 1798-5706

TIIVISTELMÄ


Luokitus: QU 83, QU 375, QW 568, QZ 360
Yleinen Suomalainen asiasana sto: hyaluronaani; melanooma; ihosyöpä; syöpäsolut; fibroblastit; entsyymit; sytokiinit; kemokiinit; solunjakautuminen; soluviestintä
To all of you, who have been part of this process.
Acknowledgements

This doctoral thesis was carried out at the Institute of Biomedicine, School of Medicine at the University of Eastern Finland between the years of 2011–2018. The work would not have been accomplished without so many colleagues whom I owe my gratitude. Thereby I want to thank all the people who helped me with my thesis.

First and foremost, I want to express my deepest gratitude to my supervisors, Docent Sanna Pasonen-Seppänen Ph.D. and Professor Emerita Raija Tammi M.D., Ph.D.; this would not have happened without your endless advice and guidance throughout these years. I admire you both for your knowledge in the hyaluronan field and the patience you had with my writing skills. I know the end was quite hectic, but you never complained. To Sanna, I want to say my thanks, for giving me the “free hands” as young scientist to conduct experiments and work independently from the beginning. Our open and close relationship made this collaboration easy and I could always come to your office to talk about anything in my mind. To Raija, I have always admired your amazing knowledge and memory, just about everything, and I wish that I could have even a fraction of that.

I also want to thank Professor Emeritus Markku Tammi M.D., Ph.D. Your knowledge of hyaluronan and its biochemical properties always amazes me. Your kind heart and warm personality made it easy to ask even the dullest questions.

I sincerely thank the official reviewers of my thesis, Professor Naoki Itano Ph.D. and Docent Kaisa Lehti Ph.D., for your extensive reviews and all the valuable comments to improve my thesis. I am also grateful to Gina Galli Ph.D. for her careful English language revision.

I am deeply grateful to all my co-authors: Docent Genevieve Bart Ph.D., Docent Kirsi Rilla Ph.D., Leena Rauhala Ph.D., Riikka Kärnä M.Sc., Antti Ropponen M.Sc., for their contribution and efforts with the publications. I also want to thank Docent Jarmo Laitinen Ph.D., Tiina Jokela Ph.D., Ashik Jawahar Deen Ph.D., Leena Rauhala Ph.D. and Lasse Hämäläinen BDM, M.Sc., for letting me be a part of your publications.

I want to express my deepest thanks to the amazing personnel at the Institute of Biomedicine. I have had the pleasure to work here many years and get to know some of you quite well. I also want to thank Docent and head of the Institute of Biomedicine Anitta Mahonen Ph.D. for enabling me to work here for so many years and take part in teaching the medical students. I also want to thank Professors Mikko Hiltunen Ph.D. and Petteri Nieminen M.D., Ph.D. for your guidance and advices for young scientists like me.

I want to express my gratitude for our “morning coffee group”; Merja Räsänen, Eija Korhonen, Kari Kotikumpu, Tiina Jääskeläinen and Silja Pyysalo. Our delightful conversations made every day start with laugh and joy.

I also appreciate the laboratory personnel in the Institute of Biomedicine. And the most special thanks goes to Riikka Kärnä M.Sc.; you were more than a coworker to me, you were my mentor in the laboratory experiments and you taught me so much. I will always cherish our friendship.

I also want to thank laboratory personnel Eija Kettunen, Eija Rahunen, Kari Kotikumpu, Arja Venäläinen and Tuula Venäläinen for your contributions to this work. Also, the secretaries of the Institute of Biomedicine; Karoliina Tenkanen and Eija Vartiainen, will get my deepest gratitude for all their help. I also want to acknowledge Arja Afflekt for keeping the “paper work” on time allowing me to defend as scheduled.
My warmest thanks also go to Professor Veijo Hukkanen M.D., Ph.D. and Michaela Nygårdas Ph.D.; you took me as an intern in your lab and taught me a lot in the field of virology and got me interested in the research field. Especially, I want to thank Michaela for your friendship; you are my idol as a gorgeous and intelligent scientist.

Moreover, to the present and former hyaluronan research group members; your friendship and our cheerful conversations during experiments made even the toughest days so funny. So, special thanks to Kai Härkönen M.Sc., Uma Thanigai Arasu M.Sc., Silja Pyysalo M.Sc., Lasse Hämaläinen, BDM, M.Sc., Tommi Paakkonen Ph.D., Ville V.T. Koistinen M.D., Ph.D., Mari Poukka M.D. and Irina Ermakova Ph.D. And students Kirsu Kainulainen B.Sc. and Elina Ero; it has been a great pleasure to get to know you. I also want to thank Docent Sanna Oikari Ph.D. for our experimental and personal discussions, Anne Kultti Ph.D. and Tiina Jokela Ph.D.; for your friendship. Katri Makkonen, Ph.D., BM and Satu Salmi BM; thank you for letting me be your “supervisor” in your MD works. Kari Törrönen Ph.D. thank you for your patience with my computer problems that made me lose my mind quite often. I also want to thank Ashik Jawahar Deen Ph.D. for your friendship, joyful personality and your and Uma’s delicious Indian cooking. I also want to express my gratitude to Docent Kirsi Rilla Ph.D., for your encouraging words during the final months of finishing my thesis.

I have also had the privilege to teach medical students with the most amazing colleagues; Leena Rauhala Ph.D. and Hanna Pohjola Ph.D.; Leena, your patience and orientation to anatomy always amazed me, and how you are able to memorize everything! Thank you also for organizing the Torsola OT-practices, it was so easy for me to just follow your example. Hanna, your palpation skills are amazing and your knowledge of anatomy and its practical use is admirable; it has been a pleasure to teach with you in the anatomical dissections and in the TLA-course.

My heartfelt thanks belong also to my great officemates, former and present. Hertta Pulkkinen Ph.D. and Hanna Siiskonen M.D., Ph.D.; you two were the first people and I will always cherish our friendship. Hertta, you taught me the importance of tabloids for relaxing. Hanna, you never stop to amaze me; how easy you make things look and get them done. During your thesis writing I was astonished how you wrote the thesis AND two papers at the same time. You two are very dear to me. I also want to thank Ayhan Korkmaz Ph.D. for your friendly personality, Raquel Melero Fernández de Mera Ph.D. for your friendships, Sami Gabbouj M.Sc., for our discussions of politics and science, Johanna Matilainen, M.Sc., for your friendship and support for me, especially at the end, and all of our discussions.

Also my heartfelt appreciation goes to Docent Virpi Tiitu Ph.D.; you are to me like the sister that I never had and a very dear friend.

As for my friends outside work, Merete Scholfield, Maria “Maikki” Timonen, Tiina Asp, Marjaana Häkkinen, Päivi Sormunen, Tiina Vuorela, Jonna Niskanen and Sari Roine; I love you all. Even when the distance is sometimes long and we haven’t seen each other for a long time, we just continue where we left off the last time. We are all from different fields, but still we never have a quiet moment; thank you for being my friends <3 Also, I especially want to thank Marjaana Häkkinen for your endless Finnish language tutoring and Finnish abstract revision.

My wholehearted gratitude also goes to my family, to my mother Tuula and father Tarmo. You have always supported me in my life and never questioned my carrier choices or any other choices in life. You have taught me the value of hard work and determination. I also want to thank my brother Reijo and his wife Päivi and your kids Tommy, Teemu and Julia; your support means a lot to me, and the interests you show towards my work. I am also grateful for your patience to travel to Kuopio now and then.
And my husband Juha; I have no words, just love towards you. You are my soul mate and my rock. We have a long history together, over 17 years, but it still feels like we just met. I am grateful of your endless support and patience with my work. I love your down to earth personality, the similar humor we share and I could not think a better father for our kid(s) (and cats). Thank you for loving me <3 for better and for worse.

The Finnish Cultural Foundation, North Savo Cancer Foundation, Kuopio University Foundation, Paavo Koistinen Foundation, Cancer Society of Finland and Doctoral Program in Molecular Medicine of The University of Eastern Finland supported this work financially.

With gratitude,

Piia Takabe

Kuopio, July 2018
List of the original publications

This dissertation is based on the following original publications:

I  Takabe P, Kärnä R, Rauhala L, Tammi M, Tammi R and Pasonen-Seppänen S. Hyaluronan fragmentation in benign melanocytes during UVB-exposure promotes TLR-4-receptor activation and proinflammatory cytokine IL-6 and chemokines IL-8, CXCL-1 and CXCL-10 expression via NF-κB activation

Manuscript


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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>4-MU</td>
<td>4 Methylumbelliferone</td>
</tr>
<tr>
<td>α-MSH</td>
<td>Alpha melanocyte stimulating hormone</td>
</tr>
<tr>
<td>α-SMA</td>
<td>Alpha smooth muscle actin</td>
</tr>
<tr>
<td>BCC</td>
<td>Basal cell carcinoma</td>
</tr>
<tr>
<td>bHABC</td>
<td>Biotinylated hyaluronan binding complex</td>
</tr>
<tr>
<td>BRAF</td>
<td>v-Raf murine sarcoma viral oncogene homolog B</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAF</td>
<td>Cancer-associated fibroblasts</td>
</tr>
<tr>
<td>CD44</td>
<td>Cluster of differentiation 44</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>CDKi</td>
<td>Cyclin dependent kinase inhibitor</td>
</tr>
<tr>
<td>CM</td>
<td>Conditioned medium</td>
</tr>
<tr>
<td>CXCL-</td>
<td>C-X-C motif ligand</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>EGF receptor</td>
</tr>
<tr>
<td>ELSA</td>
<td>Enzyme-linked sorbent assay</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>fHABC</td>
<td>Fluorescent hyaluronan binding complex</td>
</tr>
<tr>
<td>GFAT</td>
<td>Glutamine-fructose-6-phosphate aminotransferase</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>GlcUA</td>
<td>D-glucuronic acid</td>
</tr>
<tr>
<td>HAS</td>
<td>Hyaluronan synthase</td>
</tr>
<tr>
<td>HABC</td>
<td>Hyaluronan binding complex</td>
</tr>
<tr>
<td>HABR</td>
<td>Hyaluronan binding region</td>
</tr>
<tr>
<td>HARE</td>
<td>Hyaluronan receptor for endocytosis</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HMW</td>
<td>High molecular weight</td>
</tr>
<tr>
<td>HYAL</td>
<td>Hyaluronidase</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IL-</td>
<td>Interleukin-</td>
</tr>
<tr>
<td>KGF</td>
<td>Keratinocyte growth factor</td>
</tr>
<tr>
<td>LMW</td>
<td>Low molecular weight</td>
</tr>
<tr>
<td>LYVE-1</td>
<td>Lymphatic vessel endothelial hyaluronan receptor 1</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MiR</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>MITF</td>
<td>Microphthalmia-associated transcription factor</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MiR</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>MED</td>
<td>Minimal erythemal dose</td>
</tr>
<tr>
<td>MiR</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>MITF</td>
<td>Microphthalmia-associated transcription factor</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>PB</td>
<td>Phosphate buffer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed cell death protein 1</td>
</tr>
<tr>
<td>PD-L1</td>
<td>PD ligand 1</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PDGFR</td>
<td>PDGF receptor</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and Tensin homolog deleted on chromosome ten</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>RGP</td>
<td>Radial growth phase</td>
</tr>
<tr>
<td>RHAMMM</td>
<td>Receptor for hyaluronan-mediated motility</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transduced and activator of transcription</td>
</tr>
<tr>
<td>STREP-HYAL</td>
<td><em>Streptomyces</em> hyaluronidase</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumor-associated macrophages</td>
</tr>
<tr>
<td>TME</td>
<td>Tumor microenvironment</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TYR</td>
<td>Tyrosinase</td>
</tr>
<tr>
<td>TRP</td>
<td>Tyrosinase-related protein</td>
</tr>
<tr>
<td>UDP-</td>
<td>Uridine diphosphate</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>UVR</td>
<td>Ultraviolet radiation</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VGP</td>
<td>Vertical growth phase</td>
</tr>
</tbody>
</table>
1 Introduction

The occurrence of different skin cancers such as, basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and cutaneous melanoma, has significantly increased over the last decade. Even though melanoma consists of only 12% of all skin cancers in Finland (The Nordcan project, 2018), it has the highest mortality rate (Kanavy, Gerstenblith, 2011). Melanoma develops from skin melanocytes that are affected by genetic mutations and environmental factors, of which ultraviolet radiation (UVR) has most important effect. Melanoma is the most aggressive skin cancer. Unfortunately, melanoma metastasizes easily and in the metastatic phase, melanoma quickly turns to drug-resistant which reduces life expectancy (Kanavy, Gerstenblith, 2011, Sample, He, 2018, Bandarchi et al., 2010, Napolitano et al., 2018).

Ultraviolet radiation is both good and bad for human health. On one hand, it is necessary for vitamin D formation in skin, for the production of some hormones and it can even decrease depression by inducing β-endorphin production (Holick, 2016b). On the other hand, acute ultraviolet (UV) exposure can cause skin sunburn and DNA damage, and chronic UV-exposure causes skin photo-aging (wrinkling) and eventually skin cancer (Hoel et al., 2016). UVR is divided into spectrums; UVB (280–315 nm) and UVA (315–400 nm) both penetrate into the epidermis and dermis (Laihia et al., 2009). UVB radiation is the most common risk factor for melanoma. Benign melanocyte growth is tightly controlled in the epidermis and mutagenic DNA alterations induced by UVB can cause uncontrolled growth. Signaling pathways involved in melanocytes growth that are mostly mutated are v-Raf murine sarcoma viral oncogene homolog B (BRAF) and AKT, which increase the ability of melanocytes to proliferate and survive. Another common mutation in signaling pathways is in Phosphatase and Tensin homolog deleted on chromosome ten (PTEN), which is a suppressor of AKT. The loss of PTEN allows AKT to signal uncontrollably, which induces cell proliferation (Uong, Zon, 2010). While mutations occur, and melanocytes transform to malignant cells, they are still restricted to the epidermis by a basement membrane; this stage is called the radial growth phase, where melanomas are still easy to excise. Growth signals from the environment and autocrine signaling induce melanoma cells to degrade the basement membrane and invade the dermis. In this vertical growth phase, cells can disseminate metastases elsewhere in the body via the lymphatic and blood circulation (Bandarchi et al., 2010).

Hyaluronan is a high molecular weight sugar molecule, consisting of N-acetylglucosamine (GlcNAc) and D-glucuronic acid (GlcUA). Hyaluronan is produced by hyaluronan synthases (HAS1−3) at the plasma membrane and protrudes into the extracellular space during its synthesis. Hyaluronan can be bound pericellularly via its synthases or its plasma membrane receptors, such as Cluster of Differentiation 44 (CD44) (Toole, 2004). In homeostatic balance, hyaluronan exists in high molecular weight form (Laurent, 1989), but in situations such as inflammation, environmental stress or wound closure, increased hyaluronan fragmentation to lower molecular weight forms occurs (Stern, Asari & Sugahara, 2006). Hyaluronan is present from embryogenesis to adulthood and is especially abundant in the skin and is produced in the epidermis by keratinocytes and in the dermis by fibroblasts (Tammi et al., 1994). Catabolism of hyaluronan is controlled by hyaluronidases (HYAL) (Stern, 2008). As with UV radiation, hyaluronan is also good and bad for human health. On one hand, hyaluronan lubricates joints and protects the cartilage (Knudson, 1993), and during fertilization hyaluronan surrounds the secondary oocyte (Fouladi-Nashta et al., 2017) and is essential for proper heart-valve development (Camenisch et al., 2000). On the other hand, hyaluronan is associated with the aggressive nature of many types of cancers, such as breast, ovarian and pancreatic. Increased hyaluronan synthesis by tumor cells predicts poor patient prognosis in breast (Auvinen et al.,
colorectal (Ropponen et al., 1998) or prostate (Posey et al., 2003) cancers, or by stromal cells in lung adenocarcinoma (Pirinen et al., 2001), epithelial ovarian carcinoma (Anttila et al., 2000), breast cancer (Auvinen et al., 2000) or thyroid carcinoma (Böhm et al., 2002).

The interplay between cancer and stromal cells in a tumor microenvironment is essential for cancer spreading. Secreted growth factors, cytokines, chemokines and components of the extracellular matrix, such as hyaluronan, are all involved in tumor progression (Itano, Zhuo & Kimata, 2008). These factors are secreted from one cell to another (paracrine signaling) or induce signaling in the secreting cell itself (autocrine). The growth factors participate in crosstalk between different cell types at the tumor microenvironment, including; leucocytes, macrophages, mast cells, stromal fibroblasts, endothelial cells and the tumor cells (Wang et al., 2017a, Augsten, 2014). The effect of melanoma cell-associated hyaluronan or stromal hyaluronan in melanoma development and progression is still unknown. Recent studies from patient data revealed that expression of HAS1 and HAS2 decreases when melanoma progress. Furthermore, tumor hyaluronan content declines due to increased HYAL2 expression (Siiskonen et al., 2013). Decreased expression of HAS1 and HAS2 was also found to correlate with poor patient prognosis and the recurrence of melanoma (Poukka et al., 2016). However, while some progress has been made in this field, relatively little is known about the association between hyaluronan and melanoma progression and occurrence.

The present thesis aimed to study the influence of hyaluronan in different stages of melanoma; in primary melanocytes, in metastatic melanoma cells and in stromal fibroblasts. Firstly, in primary melanocytes, the aim was to study how hyaluronan metabolism is affected by UVB radiation and whether the thick pericellular hyaluronan coat protects melanocytes against harmful UVB radiation. The results showed that UVB radiation induced an inflammatory reaction via Toll-like receptor (TLR) 4 - nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) signaling and increased production of interleukin (IL) 6, IL-8, C-X-C motif ligand (CXCL) 1 and CXCL-10. The expressions of these factors were further enhanced by hyaluronan coat degradation with *Streptomyces* hyaluronidase treatment together with UVB. Secondly, in metastatic melanoma cells, the aim was to explore how increased endogenous hyaluronan synthesis, via HAS3 overexpression, affects melanoma cell behavior. These results showed that increased endogenous hyaluronan production in metastatic melanoma cells downregulates their proliferation, migration and adhesion. Thirdly, in the tumor stroma, the aim was to investigate melanoma cell secreted factors and how they affect hyaluronan synthesis and activation of stromal fibroblasts. These findings showed that melanoma cells secrete growth factors, such as platelet-derived growth factor (PDGF), which stimulate HAS2 upregulation and hyaluronan production via activation of phosphatidylinositol 3-kinase (PI3K) - AKT and p38 signaling pathways in stromal fibroblasts.

In conclusion, the results presented in this thesis broaden our knowledge on the influence of hyaluronan in melanoma development; both in the early stages of melanomagenesis as well as in the later metastatic stage. Furthermore, the results revealed the signal transduction mechanism of tumor-stroma interaction. Overall, the thesis provides novel insights for metastatic melanoma by revealing the impact of cell-cell crosstalk in melanoma tumor development and providing a new avenue for therapeutic treatment.
2 Review of the literature

2.1 MELANOMA

2.1.1 Development of cutaneous melanoma

The incidence of melanoma has increased since the 1970s (Kanavy, Gerstenblith, 2011). In Finland alone, there are over 1200 new cases each year with the incidence of skin melanoma increasing 3.7% among men and 5.7% among women in the past decade (Cancer Statistics, 2017). Melanoma is responsible for most skin cancer-related deaths (Kanavy, Gerstenblith, 2011). The most important risk factor for melanoma development is ultraviolet radiation (UVR) with up to 70% of malignant melanomas are sun-related. Nevertheless, the contribution of host and genetic factors also has a considerable influence (Sample, He, 2018, Kanavy, Gerstenblith, 2011, Bandarchi et al., 2010).

Melanoma develops from melanocytes, which are the primary pigment producing cells in the basal layer of the epidermis. Over half of new melanomas develop from a new nevus and the rest from an existing nevus (Lin et al., 2015). Melanoma has the highest rate of basal mutations (100 mutations/Mb of entire exome) mostly due to the environmental mutagen of UVR compared to other cancers (Lawrence et al., 2013). The most frequent mutation in melanoma is in the RAF pathway as the overexpression of BRAF. Other typical mutations are found in the PI3K/AKT/PTEN pathway as the loss of PTEN combined with the loss of tumor suppressor p53. Also mutations in the cell cycle control pathway CDKN2A and its coding proteins p16INK4A and p14ARF are typically mutated in melanoma (Uong, Zon, 2010). Melanoma tumors show high heterogeneity in the cell population which is driven by oncogenic signaling and microenvironmental factors. The heterogeneity of the tumor cell population makes it spread at earlier stages and enhances the development of drug-resistance (Vandamme, Berx, 2014). In the advanced metastatic stage of melanoma, the expected patient survival time is 6–12 months without effective therapy. At the moment, the most effective therapies used are targeted inhibitors against BRAF and MEK pathways and immunotherapy against programmed cell death protein 1 (PD-1) (Murali et al., 2012, Balch et al., 2001, Napolitano et al., 2018).

Immune cells, such as lymphocytes, macrophages, neutrophils and dendritic cells are responsible for normal homeostasis and inflammatory responses in skin, but they are also involved in carcinogenesis. Malignant melanoma is a highly immunogenic cancer and the infiltration of immune cells observed in histological samples, such as lymphocytes, is used in the staging of melanoma, prediction of prognosis and in the expectation of drug response (Massi et al., 2017, Elder et al., 1985, Diem et al., 2018). Pro- and/or anti-inflammatory cytokines released by the immune cells can activate neoplastic lesions and later on malignant cells. Skin-associated lymphoid tissue shows a different distribution of macrophages, dendritic cells, mast cells and natural killer cells in cutaneous melanoma, compared to normal skin (Mignogna et al., 2017). Activated M2-type macrophages, called tumor-associated macrophages (TAMs), associate with neoplastic growth and spread of the melanoma (Falleni et al., 2017) and can also be used as a prognostic marker for patient survival (Varney, Johansson & Singh, 2005). M2-type macrophages are known to secrete immunosuppressive cytokines such as IL-6 (is pro- and anti-inflammatory), IL-10, IL-23 and transforming growth factor beta (TGF-β), which can modulate the microenvironment and favor tumor progression (Falleni et al., 2017). T-cells (Nishimura et al., 1996) as well as TAMs (Huang et al., 2009) express PD-1, which mediates immune tolerance when bound to its ligand (PD-L1) (Keir et al., 2008). PD-1 was found to correlate with M2-type macrophages and reduce their phagocytic capacity against tumor cells. In colon cancer mouse models, tumor cells express the PD-L1 and binding of PD-1 from TAM inhibits the function of
TAM and increases the tumor burden (Gordon et al., 2017). Dendritic cells are the first line of defense that recruits other immune cells, such as NK cells, macrophages or leukocytes, to attack tumors cells. Chronic inflammation coupled with cytokines such as IL-1β can promote angiogenesis and the change of dendritic cell type to tumor promoting M2-like macrophages (Mak, Saunders & Jett, 2014). Similar to macrophages, mast cells can also be either anti- or pro-cancerous. The influence of tryptase and chymase, the main proteins in mast cell secretory granules, in melanoma progression has been speculated. The numbers of mast cells decrease during melanoma progression and correlate with reduced patient survival (Siiskonen et al., 2015b). It has been hypothesized that the angiogenic factors secreted by mast cells induce tumorigenic potential in the neoplastic lesions, but not in the metastatic tumors (Biswas et al., 2014).

Melanocytic cells /melanoma cells are in constant interaction with surrounding cells such as macrophages, lymphocytes and fibroblasts. All these cells secrete several soluble factors, which modify target cells and the whole tumor microenvironment, in addition to UVR. Thereby, melanoma cells and the whole tumor microenvironment (TME) are highly dynamic, making melanoma treatment challenging.

**Staging of melanoma**

Melanomas are generally classified as; superficially spreading, which accounts 50–75% of melanomas, nodular melanomas (15–35%), lentigo maligna melanomas (5–15%) and acral lentiginous melanomas (5–10%). More rare and uncommon types are desmoplastic melanoma and miscellaneous melanoma (Bandarchi et al., 2010, Mihm, Lopansri, 1979, McGovern et al., 1973). TNM-staging is used to classify the stage of melanoma. T (tumor) describes the depth of the tumor, N (node) how far it has spread to the surrounding lymph nodes and M (metastasis) if there are existing distant organ metastases. Breslow’s index describes the thickness of the melanoma and evaluates the actual thickness of the tumor; the index is measured from histological sections with a light microscope. Non-invasive melanomas (melanoma in situ) are located only in the epidermis. The thickness of the superficial melanoma is <1 mm, intermediate tumors are between 1–4 mm and deep melanomas are >4 mm (Tokgoz et al., 2012). Both classifications are used in the clinic. Early phase diagnosed melanoma can be surgically excised, but when melanoma has metastasized it is more difficult to treat, especially if it has acquired resistance to chemotherapy (Garbe et al., 2010). Radial and vertical growth phase also describes melanoma progression (Fig. 1). Melanoma in situ is in radial growth phase, this phase may have some microinvasive character where the melanocytic cells are present in the superficial papillary dermis. Vertical growth phase melanomas have entered the dermis and may already have sent metastatic cells to the nearby lymph nodes (Bandarchi et al., 2010).
2.1.2 Skin and its cellular components

Consisting of 15% of the total adult body weight, skin is the largest organ of the body. Skin (Fig. 2) consists of three layers (top to bottom): the epidermis, the dermis and the hypodermis. The epidermis contains several cell types such as keratinocytes, melanocytes, Langerhans cells and Merkel cells. In the epidermis, keratinocytes are the main cell type, consisting of 90–95% of the total cell population. The epidermis is divided into continuous cell layers, each having its own distinct features. The bottom layer, called the basal cell layer, is a single cell layer thick followed by the prickle-cell layer, also known as stratum spinosum, consisting of 5–15 cell layers. Above the prickle-cell layer is the granular cell layer, stratum granulosum of 1–3 cell layers. The top layer of the epidermis is the horny layer or cornified layer, stratum corneum, which contains 5–10 cell layers (Kanitakis, 2002). The dermis mainly contains fibroblasts and the extracellular matrix they produce. Other cell types in the dermis include leucocytes, neural cells and vascular cells. The hypodermal layer below the dermis mainly consists of adipocytes (Sorrell, Caplan, 2004).
Figure 2. The skin and its layers. The epidermal layer consists mainly of keratinocytes, the dermal layer of fibroblasts and the hypodermis of adipocytes. Pigmented melanocyte cells, as well as mechanoreceptor Merkel cells, are located at the stratum basale, whereas Langerhans cells are found in all the epidermal strata. The dermal layer consists of abundant extracellular matrix molecules such as hyaluronan, collagens and elastin (modified from Gaur, Dobke & Lunyak, 2017).

**Keratinocytes**
Keratinocyte morphology in the epidermis varies depending on the layer they are situated. Keratinocytes in the basal layer are columnar or cuboidal and are aligned vertically above the underlying basement membrane. In the prickle-cell layer keratinocytes are larger and their shape is more polygonal. Cell shape changes gradually from polygonal cells in the prickle-cell layer to thin, flattened cells in the granular layer. In the granular cell layer, cells contain numerous dark keratohyalin granules, where profilaggrin, a precursor of filaggrin, is stored. During granular cell differentiation to cornified cells, profilaggrin is proteolyzed to filaggrin monomers, which aggregate with keratin filaments from cornified cells to form keratin patterns (Ishida-Yamamoto, Igawa & Kishibe, 2018). The cells in stratum corneum, corneocytes, are flattened, squamous-like, keratin-filled, devoid of cell organelles, and eventually shed from the skin surface (Kanitakis, 2002).

**Langerhans and Merkel cells**
Langerhans cells are found in all stratified epithelia and are located in the upper epidermal cell layers. In contrast, melanocytes are found in the basal layer. Langerhans cells are dendritic antigen presenting cells for naïve T-cells in the skin (Bandarchi et al., 2010, Girolomoni et al., 2002). Merkel cells express both neuroendocrine and epithelial features; they are sensory receptors that act in mechanoreception which are found in the basal layer of the epidermis as well as in the sheath of hair follicles (Lacour et al., 1991).

**Fibroblasts**
The precursors of dermal fibroblasts originate from the dermomyotome. These progenitor cells form two lineages of dermal fibroblasts; papillary and reticular. Papillary fibroblast lineage forms dermal papillary fibroblasts and hair follicle fibroblasts, whereas reticular progenitors form reticular fibroblasts and pre-adipocytes to the hypodermis (Thulabandu, Chen & Atit,
Papillary dermis, which is just below the epidermis, contains microvasculature and neural components as well as characteristic extracellular matrix (ECM) produced by the fibroblasts. Papillary ECM consist of loose irregular collagen I and III fibers, nonfibrillar collagen XII and XVI, decorin and tenasin C. The reticular dermal layer extends from this papillary layer up to the hypodermis and contains deeper vascular plexus and hair follicles. ECM in the reticular dermis is more organized to dense fiber bundles of collagen types of I, III, XIV, elastin fibers, versican and tenasin X (Sorrell, Caplan, 2004). Hyaluronan is abundant in the ECM of the papillary dermis (Röck, Fischer, 2011), but its quantity is also high in the reticular dermis (Tammi et al., 1994).

**Immune cells of the skin**
The skin comprises abundant populations of immune cells, including dendritic cells, macrophages, resident memory T-cells, mast cells and innate lymphoid cells. In addition, host immunity recruits infiltrating T-cells, monocytes, neutrophils, basophils and eosinophils. These skin-associated lymphoid tissue cells have a prominent role in normal homeostasis in the skin, and in the inflammatory reactions as well as tumorigenesis (Mignogna et al., 2017, Ono, Kabashima, 2015).

**2.1.3 Melanocytes and melanin unit**
Melanocytes are of neural crest origin and migrate in the basal layer of the epidermis (Schiaffino, 2010); these cells are distributed among the keratinocytes and their density varies depending on the region of the body from legs and arms (1,000 per mm²) to face and forehead (2,000 per mm²) (Szabo, 1954). Melanocytes produce a melanin-pigment to protect the skin, especially epidermal keratinocytes, against the harmful effects of UVR (Mohania et al., 2017). Melanin pigments, eumelanin and pheomelanin, are large biopolymers (Schiaffino, 2010, Prota et al., 1998) which are produced in the melanocytes in specialized subcellular organelles called melanosomes (Bandarchi et al., 2010); and are further distributed to the keratinocytes for photoprotection (Hearing, 2011). Melanosomes undergo maturation steps from stage I to stage IV. Stage IV melanosomes are fully mature and are covered by the eumelanin pigment. In the end process of melanogenesis, prostaglandin E2 (PGE2) and alpha melanocyte-stimulating hormone (α-MSH) mediate the transfer of mature melanosomes via dendritic processes to enclosed keratinocytes via PAR-2 receptors on the keratinocyte plasma membrane (Bandarchi et al., 2010, Marks, Seabra, 2001, Ma et al., 2014a). In keratinocytes, melanosomes form a protective cap on the top of the nucleus against harmful effects of UVR.

Melanocytes in the basal layer of the epidermis extend their dendritic processes to surrounding keratinocytes, approximately 36. Together they form the epidermal melanin unit (Fitzpatrick, Breathnach, 1963). The homeostasis in the epidermal melanin unit is tightly regulated by the keratinocytes (Turner et al., 2006), but also by the melanocytes. Signaling through paracrine growth factors, intracellular signaling pathways and communication via cell-cell adhesion and cell-matrix adhesion are all crucial for melanocyte growth (Haass, Smalley & Herlyn, 2004). The adhesion of melanocytes to surrounding keratinocytes is mediated via E-cadherin, which controls the interaction of cells in the melanin unit (Tang et al., 1994). The imbalance gives melanocytes an advantage of dysregulation of the melanocyte homeostasis. Triggers, such as loss of E-cadherin (Li et al., 2001) and increased β-catenin signaling (Heasman et al., 1994), can lead to continuous proliferation of the melanocytes via autocrine growth factors. The proliferation causes the constitutive activation of signaling cascades such as mitogen activated protein kinase (MAPK) and PI3K, leading to uncontrolled life span and escape from keratinocytes control (Haass, Herlyn, 2005).

**Melanosome biogenesis and microphthalmia-associated transcription factor**
Melanosome maturation begins from the so-called pre-melanosome stage (early stage I), which lacks melanin and consists of only vesicular structures with internal membranes. Stage II is still
referred to as pre-melanosome, it differs from stage I only with its elongated structures with internal striations, whereas polymerized melanin accumulates at stage III and maturation begins. Mature melanosomes (state IV) are filled with melanin together with specific proteins; tyrosinase (TYR), tyrosinase-related protein 1 (TRP1) and Pmel17 (Yamaguchi, Hearing, 2009). The earliest publications of melanin biosynthesis dates back to early the 20th century. Tyrosinase is the key enzyme in the synthesis of melamins (Raper, 1927). Regulation of pigment production, melanocyte differentiation and proliferation are regulated by the microphthalmia-associated transcription factor (MITF). α-MSH binding to melanocortin 1 receptor (MC1-receptor) activates cAMP production and activation of the melanocyte-specific isoform of MITF, MITF-M, and phosphorylation of the transcription co-factor CREB (Price et al., 1998). Activation of CREB and transcription cofactors such as p300, c-Fos and P/CAF (Sato et al., 1997) initiate the transcription of TYR, TRP1 and dopachrome tautomerase (DCT) leading to melanin production (Levy, Khaled & Fisher, 2006).

Highly polymorphic MC1-receptor gene is located on chromosome 16q24.3 and over 80 different variants have been described. MC1-receptor variations results in a shift from eumelanin (black brown) expression towards pheomelanin (yellow-red), and this associates with a red hair color phenotype (Schiøth et al., 1999, Gerstenblith et al., 2007, Mountjoy et al., 1992). Eumelanin is the most common melanin in dark skin and hair. Eumelanin acts on reducing the accumulation of the ultraviolet (UV) induced photoproducts, while pheomelanin induces free radical formation after UV and generates UV-induced DNA damage (Sample, He, 2018, Schiaffino, 2010). Mutations in genes regulating pigment synthesis is related to human pigimentary diseases such as albinism, vitiligo, oculocutaneous albinism type 1–4, piebaldism, Hermansky-Pudlak syndrome, Hirschsprung’s disease and Waardenberg’s syndrome (Yamaguchi, Hearing, 2014). Mutations in MC1-receptor genes or inactive receptor function lead to deficiency of eumelanin production and increases the susceptibility of melanoma (Mitra et al., 2012).

MITF is required for melanocyte development from neural crest origin (Hemesath et al., 1994); this regulates melanocyte proliferation (Konyukhov, Kindyakov & Malinina, 1994), differentiation (Bentley, Eisen & Goding, 1994) and the genes involved in pigmentation (Hemesath et al., 1994). MITF is expressed in nine isoforms, which all have a DNA binding/dimerization domain and two transactivation domains. MITF-M, the melanocyte specific form, is involved in pigment synthesis and it regulates melanin synthesis enzymes TYR, TYRP1 and DCT and the synthesis of PMEL, MLANA, RAB27A (Levy, Khaled & Fisher, 2006, Du et al., 2003, Yasumoto et al., 1997). It is also involved in DNA replication and repair, cell proliferation and mitosis (Strub et al., 2011) by regulating the transcription of cell cycle proteins such as CDKN1A, CDKN2A, and CDK2, and survival proteins such as BCL2, HIF1A and MET (Cheli et al., 2010). It is postulated that the level of expressed MITF activity, defines its action. High activity levels enhance melanocyte differentiation, medium activity levels foster proliferation, low levels promote an invasive stem cell-type phenotype, while MITF deficiency can lead cell senescence or death (Goding, 2011). Post-transcriptional modifications such as phosphorylation by MAP-kinase proteins extracellular signal-regulated kinase 2 (ERK2), ribosomal S6 kinase (RSK), glycogen synthase-3β (GSK3β) and p38, can modulate MITF’s transcriptional activity in response to environmental pressure (Levy, Khaled & Fisher, 2006).

**Microphthalmia-associated transcription factor in melanomagenesis**

MITF acts as an oncogene, which is often amplified or overexpressed in human malignant melanomas, but its effect is rather controversial. MITF-driven changes in melanocytes are linked to epithelial-mesenchymal transition (EMT), proliferation, cell cycle arrest and senescence. ZEB2, a transcription factor regulating EMT, was shown to be an upstream regulator of MITF and low ZEB2 expression was linked to shortened melanoma-specific survival (Denecker et al., 2014). MITF also regulates tumor suppressor p16INK4a maintaining its expression (Loercher et al., 2005), and p16INK4a mutations are frequently found in melanoma
patients (Piccinin et al., 1997). A protein kinase involved in cell cycle control, cyclin dependent kinase (CDK) 2, is regulated by MITF. Loss of MITF activity leads to increased CDK2 expression and uncontrolled cell cycle regulation (Du et al., 2004). Growth factors that influence cell growth, such as TGF-β, affect MITF activity dose-dependently and inhibit TYR and TRP protein synthesis (Kim, Park & Park, 2004) a similar effect was discovered with fibroblast growth factor 21 (FGF21) (Wang et al., 2017b). Recently Najem et al. (2017) identified MITF/Bcl-2 and p53 as key pathways in MEK inhibitor resistance in NRAS-mutant melanomas (Najem et al., 2017). MicroRNA26a (miR-26a) was shown to target MITF expression by directly binding to the MITF gene and reducing its expression in MITF-high melanoma cells. MITF downregulation by miR-26a results in reduced melanoma cell invasiveness and proliferation (Qian, Yang & Yang, 2017); this opens new potential targets in melanoma therapy.

2.1.4 Mutations in melanoma

During melanoma development, genetic mutations, microenvironmental signals and reversible changes give the melanocytes an advantage to cellular plasticity. The heterogeneity of melanoma cells makes them difficult to treat and the cells easily disseminate from the initial lesion and become drug-resistant (Vandamme, Berx, 2014). Common risk factors for melanoma development are sunburns in childhood and young adulthood, light skin color, red hair and a high number of moles on the body (Holick, 2016a). The first changes in cellular heterogeneity are affected by factors that induce EMT. In terms of melanoma, the first change that occurs in melanocytes is the loss of E-cadherin expression. E-cadherin is involved in cell-to-cell adhesion and it limits cellular motility in melanocytes (Vandamme, Berx, 2014, Li et al., 2001). Decreased E-cadherin expression increases β-catenin signaling, which in turn alters gene expression and favors malignant transformation (Lee, Herlyn, 2007). Invasion through the basement membrane is the stage where radially growing cells (RGP) turn to the vertically growing phase (VGP). Cells in VGP start to express αvβ3 integrin, which is linked to the loss of E-cadherin expression in transformed melanocytes; these changes are controlled by the PTEN/PI3K pathway (Hao et al., 2012, Albelda et al., 1990).

Mutations in cell cycle regulator CDKN2 in melanoma

In addition to microenvironmental pressures, oncogenic mutations and loss of tumor suppressors are driving forces for melanomagenesis. The first common mutations found in melanoma patients were linked to chromosome 9p21-p22 (Dracopoli et al., 1987, Fountain et al., 1992), which also associates with familial melanomas (Cannon-Albright et al., 1992). This region encodes tumor suppressor gene CDKN2 (Kamb et al., 1994) which regulates CDK4 and CDK6 via p16 protein and controls the cell cycle (Serrano, Hannon & Beach, 1993). Mutations in this p16 gene were shown to correlate with melanoma development and progression (Piccinin et al., 1997). The CDKN2A locus encodes cell cycle regulation protein p14 (Stott et al., 1998), which was shown to negatively regulate tumor suppressor protein p53 function in metastatic melanoma together with p16 (Sauroja et al., 2000). Mutations in p53 have been shown only in 20% of melanomas (Mar et al., 2013), but loss of p53 in BRAFV600E mutated melanocytic cells induces cell proliferation (Yu et al., 2009), together with abnormal expression of its downstream pro-apoptotic target genes, such as Bax (Avery-Kiejda et al., 2011).

BRAF mutations in melanoma

The BRAF gene is the most mutated gene in melanoma; mutation is found in approximately 60% of melanoma patients. BRAF regulates the RAS-RAF-MEK-ERK-MAP kinase pathways that regulate the growth and proliferative responses in cells. The mutation was first discovered by Davies et al. (2002) and is localized to exon 15: T1796A, where a valine is substituted by glutamic acid (V600E) (Davies et al., 2002). In melanocyte homeostasis, a stimulus from α-MSH binds to the MC1-receptor and PKA via cAMP mediates the signal to CRAF, an isoform of the RAF protein. This signaling route maintains the normal balance in melanocyte proliferation,
growth equilibrium and melanin synthesis (Hunt et al., 1994). An imbalance in melanocyte homeostasis occurs when mutations in the RAS pathway switch from CRAF isotype to BRAF overactivation. BRAF itself can activate the MEK pathway after stimulus from the α-MSH-MC1-receptor-complex and this induces proliferative signaling (Dumaz et al., 2006). Increased microRNA (MiR-21) signaling was also observed in the transition of benign melanocytic lesions to malignant melanoma. MiR-21 is associated with BRAF and NRAS mutations and induced sustained proliferation, genetic instability, increased oxidative stress and decreased apoptosis (Melnik, 2015). BRAF and MEK inhibitors are the most frequently used drugs in melanoma therapy, however melanoma cells easily gain resistance to BRAF inhibitors due to cellular heterogeneity. Some of the tumor cells may acquire a mechanism via alternative splicing of BRAF, NRAS/KRAS mutations and MEK1/2 mutations to reactivate MAPK signaling, leading to inhibitor resistance (Nazarian et al., 2010).

**PTEN mutations in melanoma**

Signaling through the MC1-receptor also controls PTEN expression and the suppression of PI3K/AKT signaling (Cao et al., 2013). PTEN is an inhibitor of the cell growth signaling pathway PI3K/AKT (Maehama, Dixon, 1998). Decreased expression of PTEN is found in almost 50% of primary melanoma tumors (Wu, Goel & Haluska, 2003), which leads to constant activation of AKT signaling. This pathway itself is not enough to induce uncontrolled growth of cells, unless coupled to the loss of tumor suppressor p53 (Chen et al., 2005). UVB-exposure stimulates the formation of MC1-receptor/PTEN complex, which suppresses AKT activation (Cao et al., 2013). People with fair skin, red hair and poor ability to tan have MC1-receptor variants which are not able to interact with PTEN after UVB-exposure. Therefore, these individuals are more prone to melanoma compared to people with the normal MC1-receptor form (Cao et al., 2013, Raimondi et al., 2008, Kennedy et al., 2001). Loss of PTEN and increased AKT signaling can inactivate Bcl-2 family member BAD, which is involved in the control of apoptosis (Datta et al., 1997). AKT signaling is also found to control the telomerase activity by phosphorylating of hTERT, which in turn enhances AKT activity and gives cells the benefit of uncontrolled growth regulation (Kang et al., 1999).

In melanoma development, multiple mutations in genes controlling cell cycle are needed to transform benign melanocytes to tumor cells. Concurred mutations in BRAF and PTEN results in constant activation of proliferative ERK signaling and the escape of apoptosis and uncontrolled telomerase function, mediated via AKT pathway. Recently a link between these pathways was found to contribute to immunotherapy resistance (Jain et al., 2017). Abelson non-receptor tyrosine kinases (Abl), Abl and Arg, can co-operate in parallel in both of the signaling pathways BRAF^{V600E}/EKR and PI3K/AKT, and they are highly active in melanomas (Jain et al., 2017). New whole tumor sequencing techniques have also revealed the importance of mutations in the promoter region of different genes. In active promoter regions, sequence TTCCG is the most frequently mutated site, which is vulnerable to UV-induced mutagenesis and characteristic to melanoma tumors (Fredriksson et al., 2017). These new studies point out the complexity of melanoma and the mutations causing it, but also reveal new targets for drug therapy.

### 2.2 ULTRAVIOLET LIGHT

#### 2.2.1 Spectrum of light

The wavelength of visible light is between 400–780nm, which partly overlaps the spectrums of UV and near infrared. There are three categories of UV radiation, UVC (100–280 nm), UVB (280–315 nm) and UVA (315–400 nm), the latter overlaps with visible light (Sliney, 2016).
Skin absorbs UV and visible light; this depends on the light-type wavelength and how far it penetrates the skin. The epidermis absorbs most of the UVB radiation, while UVA is able to go further to the upper layers of the dermis, and visible light easily reaches the dermal skin layer (Laihia et al., 2009).

2.2.2 The biological effects of UV-light

Biologically, UV radiation has benefits and disadvantages on human health. The minimal erythemal dose (MED) is defined as the lowest dose of UVR that can cause mild redness. MED depends on the skin type, but for fair skin it is 200 J/m². Repeated exposure to UVR causes tanning of the skin (melanin synthesis) and thickening of the epidermis (hyperplasia) (Laihia et al., 2009). UVR-induced epidermal thickening is more pronounced in people with fair skin than in people with a darker complexion. Epidermal hyperplasia seems to be a non-pigmentary protective mechanism in individuals with low skin melanosome content (Hennessy et al., 2005).

A major benefit of UVR is the induction of vitamin D production and thus the maintenance of calcium homeostasis. UVB radiation induces a photochemical reaction which leads to the formation of cholecalciferol (D3-vitamin) from 7-dehydrocholesterol in the epidermis by keratinocytes (Lehmann, 2009). Vitamin D3 is further modified to a biochemically active form, first in the liver and thereafter in the kidneys. Active vitamin D is important in the absorption of calcium from the small intestine and its deficiency can lead to rickets in children (Holick, 2016a, Laihia et al., 2009, Schuch et al., 2017). Other benefits from UVR include UVA-induced ROS-mediated nitric oxide generation, which reduces blood pressure and hence lowers the risk for heart disease (Young, Claveau & Rossi, 2017). UVR also stimulates β-endorphin production (Jussila et al., 2016) which reduces the risk for depression but can also cause addiction to tanning (Fell et al., 2014). UVR also increases keratinocyte adenocorticotropin hormone production which helps to modulate immune responses (Holick, 2016b). The adverse, acute effects of UVR include erythema (sunburn), DNA damage and suppression of acquired immunity by preventing the activation of T-cells (Norval, Halliday, 2011). The chronic effects of UVR include photoaging (dermatoheliosis) and eventually photocarcinogenesis (skin cancer). Excessive exposure to UVB is the main factor for skin cancers such as cutaneous malignant melanoma and non-melanoma skin cancers such as, basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) (Young, Claveau & Rossi, 2017, Hoel et al., 2016). UVR induced reactive oxygen species (ROS) formation increases matrix metalloproteinase (MMP) expression and dermal ECM degradation. MMPs degrade collagens and elastin, which give the skin strength and elasticity. In the long term, degradation of dermal ECM causes skin wrinkling, dehydration and hyperkeratosis (Pittayapruek et al., 2016, Laihia et al., 2009). In addition, photoaging affects hyaluronan degradation in skin (Kurdykowski et al., 2011).

UVA radiation causes the formation of free radicals that can damage the DNA in skin cells by generating the crosslinking of pyrimidine bases between thymine and cytosine. UVA also penetrates to the dermis causing the crosslinking of the collagen-elastin network that results in skin damages and wrinkling (Holick, 2016a). UBV, on the other hand, forms uracil dimers, cyclobutane pyrimidine dimers and 6,4-pyrimidine-pyrimidones to double stranded RNA (dsRNA). These dimers cause premutagenic alterations to DNA and can lead to inhibition of DNA polymerase, cell replication arrest, increased frequency of mutations and eventually carcinogenesis (Schuch et al., 2017, Holick, 2016b). UVB is absorbed by epidermal proteins, such as melanin pigment and urocanic acid which protects DNA from damages. Melanin pigments, secreted by melanocytes, absorb UV wavelengths between 300–370 nm, thereby protecting the cells against UVB and UVA radiation (Holick, 2016a, Laihia et al., 2009).

2.2.3 UV-induced stress and cell signaling

UVA and UVB exposure generates electromagnetic energy, which is absorbed by cellular chromophores such as DNA, porphyrines, urocanic acid and aromatic amino acids. These energized chromophores react with molecular oxygen generating ROS such as superoxide (O₂⁻).
and hydroxyl radicals (HO·) (free radicals) or hydrogen peroxide (H₂O₂) and oxygen (O₂) (non-radical compounds). Intracellular control mechanisms regulate the level of ROS via enzymatic and non-enzymatic antioxidants. UVR triggers the stabilization of antioxidant response factor nuclear factor E2-related factor 2 (NRF2), which activates the antioxidant response by binding to response elements in gene promoter areas. The overactivation of antioxidants may lead to cellular damage, such as apoptosis and necrotic cell death, when the antioxidant defense mechanism is overwhelmed, but ROS also cause oxidative stress by misbalancing the equilibrium of pro-oxidants and antioxidants (Sample, He, 2018, Sander et al., 2004, Xu, Fisher, 2005).

UVR has been reported to activate cell surface receptors either directly or via ROS. The most established receptor activated by UVR is the epidermal growth factor receptor (EGFR) (Correia et al., 2014, Ley, Ellem, 1992). ROS-induced H₂O₂ causes autophosphorylation of EGFR and this leads to downstream activation of ERK1/2 and RAS/MAPK-inducer Shc. Shc acts in the survival pathway in UVB-exposed skin keratinocytes (Peus et al., 2000). Several other receptors are proposed to activate via a UV-induced mechanism, these include; insulin receptor (Lewis et al., 2008, Coffer et al., 1995), c-Ret (Kato et al., 2000), cytokine receptors for tumor necrosis factor α (TNFR1/R2), interleukin 1 receptor (IL-1R), death receptor Fas, and growth factor receptors for fibroblast growth factor (FGF), hepatocyte growth factor (HGF) and PDGF (Muthusamy, Piva, 2010, Xu, Fisher, 2005). The activated signaling routes involve MAPK-ERK, p38 and c-Jun N-terminal kinases (JNK), PI3K/AKT and NF-κB. These signaling routes lead to the production of proinflammatory cytokines such as IL-1β and TNF-α (Muthusamy+Piva, 2010). In skin keratinocytes, UVB induces TNF-α secretion activates NF-κB signaling and its subunit RelA nuclear localization, which is able to increase the survival of transformed cells (Muthusamy, Piva, 2013, McNulty, Tohidian & Meyskens, 2001).

Recently, it was also shown that UVR (275−380 nm) induces the activation of Transient receptor potential type 1 (TRPV1) in HaCaT keratinocytes. TRPV1 activation mediated MMP-1 expression as well as secretion of proinflammatory cytokines IL-6 and TNF-α. These effects were inhibited by TIP, a synthetic peptide against TRVP1. TIP was also used in UV-irradiated mouse models, where it attenuated erythema, Mmp-1, Mmp-2 and Mmp-9 expression and IL-6 and IL-8 production, indicating its potential implications in UV-induced inflammation and photoaging (Kang et al., 2017).

MAPK pathway
Pathways activated after UVR are vital for cell survival through UV-induced stress. JNK and p38 are stress-response kinases and have shown to activate after UVR-exposure, as well as EGFR-ERK1/2 signaling (Xu, Fisher, 2005). ROS-induced H₂O₂ causes the activation of p38 (Ren et al., 2016, Zhu et al., 2015, Rauhala et al., 2013, Xu, Voorhees & Fisher, 2006, Peus et al., 1999). This occurs immediately after UV-exposure, probably independently of EGFR activation and other MAP-kinases. For example, in dermal fibroblasts, phosphorylation of p38 is induced directly by ROS after UVA radiation (Le Panse, Dubertret & Coulomb, 2003). p38 activation is involved in the regulation of cell cycle checkpoints G1/S and G2/M following DNA damage and in the regulation of mitotic transit and cytokinesis in cell division (Tormos et al., 2013, Thornton, Rincon, 2009). In keratinocytes, p38 activates p53 protecting the cells against UV-induced apoptosis (Chouinard et al., 2002). Furthermore, UV-induced p38 signaling mediates either transcription of Bcl-XL, a member of anti-apoptotic Bcl-2 family (Bachelor, Bowden, 2004) or pro-apoptotic Bax in keratinocytes (Van Laethem et al., 2004), depending on the UV dose and on the amount of damage in cells.

JNKs exist in three isoforms (JNK1−3) and their level of activation depends on the cell type. In keratinocytes, UVA plays a bigger impact in activating JNK signaling compared to UVB, whereas in macrophages, UVB induces higher JNK activation (Sodhi, Sethi, 2003, Chouinard et al., 2002). Karin et al. (2005) proposed that the JNK signaling route is responsible for TNF-α secretion and prolonged signaling which induces malignant chances in cells (Karin, Gallagher,
Both p38 and JNK induce activator protein 1 (AP-1) activation and cyclooxygenase-2 (COX-2) expression which mediates the UV-induced inflammatory response (Cho et al., 2005), and also controls UV-induced melanin synthesis in melanocytes (Kim et al., 2012). Of the different ERK isoforms (ERK1/2, ERK3/4, ERK5 and ERK7/8) ERK1/2 is the best-characterized isoform in UV responses. ERK1/2 phosphorylation is triggered by upstream activation of RAF-1, BRAF or ARAF, which in turn activate MEK1 and MEK2 leading to ERK phosphorylation. ERK1/2 downstream targets are Elk 1, c-Fos and c-Myc (Muthusamy, Piva, 2010). ERK1/2 activation by UV prevents apoptosis by inhibiting the activation of caspase 3 (He, Huang & Chignell, 2004). ERK signaling enhances cells proliferative processes and its sustained activation has an important effect in melanomagenesis, especially in BRAF mutated melanomas, where BRAF works upstream of ERK1/2 (Satyamoorthy et al., 2003).

PI3K/AKT pathway
PI3K/AKT is an important pathway for cell survival and its activation prevents apoptosis in UV-exposed keratinocytes. The PI3K/AKT pathway can be activated via UV-EGFR activation or directly by UV (Xu, Fisher, 2005). The PI3K-AKT pathway may also lead to the activation of transcription factors, such as AP-1 transcription family members JUN, JDP, FOS/FRA or MAF; these factors mediate the transcription of MMP2, MMP-14, MUC-18 (Bosserhoff et al., 2014, Lopez-Camarillo et al., 2012) and cancer-relevant genes such as R-RAS, PKC-α, PDGF-C (Schummer et al., 2016).

RAS-PI3K signaling increases AKT activation which leads to transcription of cell survival genes coupled with the activation of transcription factor mTOR. mTOR activation is involved in cell proliferation, growth and survival, angiogenesis and metabolic processes such as glucose uptake as well as antiapoptotic signaling and tumorigenesis (Yajima et al., 2012, Manning, Cantley, 2007). mTOR exists in two functional complexes, mTORC1 and mTORC2. mTORC1 contains the activation site for AKT-mediated phosphorylation and regulates cell growth by controlling ribosome biogenesis, and protein and lipid synthesis (Guertin, Sabatini, 2007). An overactive PI3K-AKT-mTOR pathway has been detected in PTEN mutated melanomas, contributing to tumor progression (Chan et al., 2017). Furthermore, PI3K-AKT-mTOR pathway activation is involved in BRAF inhibitor resistance by activating alternative survival pathways in melanoma cells (McCubrey et al., 2006). UVB-induced mTOR activation in keratinocytes controls pro-survival signaling (Carr et al., 2012); inhibition of mTORC1 reduces cell proliferation and cell cycle progression, whereas mTOR deletion inhibits mTORC2 target AKT phosphorylation and increases keratinocytes apoptosis. These findings indicate the complementary roles for mTORC1 and mTORC2 in UVB-induced cell signaling (Carr et al., 2012).

Isoforms of AKT are expressed in different layers of the epidermis; AKT1 is expressed in the granular layer, while AKT2 is expressed in all layers, but phosphorylated only in the lower suprabasal layers. In normal adult skin, AKT2 expression is low (O'Shaughnessy et al., 2007b) and AKT1 is the main isoform which regulates terminal differentiation of the keratinocytes. AKT1 expression is decreased in SCC, while the expression of AKT2 is increased; this suggests a tumor suppressive impact for AKT1 compared to an oncogenic effect for AKT2 in skin (O'Shaughnessy et al., 2007a). In mouse skin, acute and chronic UVR irradiation downregulates AKT1 expression and upregulates AKT2. Inhibition of mTOR with rapamycin decreases AKT2 signaling and increases AKT1 signaling in UV-exposed keratinocytes or skin samples. AKT1 phosphorylation mediates cell recovery after UV-exposure, indicating opposite effects of AKT1 and AKT2 in mTOR signaling (Sully et al., 2013). Cross-talk between PI3K-AKT-mTOR and RAS-RAF-MEK-ERK pathways occurs in melanoma (Jokinen, Koivunen, 2015). Consequently, inhibition of one pathway may allow the other pathway to take over, especially when using single-agent therapies. Therefore, dual targeting these two main pathways might promote patient therapy (Jokinen, Koivunen, 2015).
**NF-κB pathway**

NF-κB activation regulates the expression of inflammatory cytokines induced by ROS, UV, γ-irradiation or TNF-α and IL-1β (Tormos et al., 2013). NF-κB exists in dimer complexes with Rel-family proteins of p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1) and p52/p100 (NF-κB2). The inhibitory proteins of NF-κB, IκBs, bind to the cytoplasmic complex of NF-κB/Rel. IκBs are phosphorylated by IκB kinases (IKK) to activate the NF-κB/Rel complex. As a result, IκB is degraded from the complex by proteasome 26S and NF-κB can be translocated to the nucleus and activate the transcription of target genes. UVR alone or ROS produced by UVR has been shown to inhibit IκB directly and thereby activate NF-κB signaling (Xu, Fisher, 2005, Hayden, Ghosh, 2004, Wu et al., 2004). UV-induced NF-κB activation induces the secretion of IL-1β, TNF-α, IL-6 and vascular endothelial growth factor (VEGF) in keratinocytes, fibroblasts and Langerhans cells (Abeyama et al., 2000). Other signaling pathways, such as PI3K/AKT2, can also activate NF-κB signaling. UVC-irradiation causes NF-κB activation and protects cells from apoptosis via PI3K/AKT2 signaling (Yuan et al., 2002) or by suppressing p38α expression via MiR-125b upregulation (Tan et al., 2012). Therefore, NF-κB might be the key antiapoptotic/prosurvival factor in UVA- and UVB-induced melanocytes, since its inhibition is able to induce the release of cytochrome c from the mitochondria, caspase activation and nuclear fragmentation in melanocytes after UVR (Wäster, Rosdahl & Ollinger, 2014).

### 2.2.4 UV-induced apoptosis and cell cycle arrest

Apoptosis is a cellular defense mechanism to remove damaged or transformed cells, but it is also a part of normal cell regulation (programmed cell death). The ability of the cells to balance between proliferation and apoptosis, maintains normal tissue homeostasis. The imbalance in tissue homeostasis, an alteration in either proliferation or apoptosis, has a major impact in tumorigenesis. Failure in the protective mechanisms, such as growth arrest followed by DNA repair, cell death via apoptosis or the malfunction of tumor suppressor gene pathways, results in abnormal cell proliferation and even tumorigenesis later in the process. ROS, DNA damage, activation of tumor suppressor p53-gene, mitochondrial damage, trigger of cell death receptors, are shown to be activated via UV-induced apoptosis. DNA damage repair during cell cycle arrest is mediated via p53 to DNA damage sensors ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) proteins. These proteins recognize single-strand DNA breaks and phosphorylate checkpoint kinase 1 (Chk1) and activate checkpoints at the G1, S and G2/M phases to ensure controlled repair and proliferation. Cyclins and CDKs are the regulatory mechanism in cell cycle control, with each having a specific time of appearance and kinase activity (Sample, He, 2018, Sander et al., 2004, Chan, Yu, 2000, Kulms, Schwarz, 2000, Shackelford, Kaufmann & Paules, 1999).

In primary melanoma cells, UVB-induced cell cycle arrest at G1, S and G2/M phases, is mediated through the association of p21 with cyclin E/CDK2 and cyclin A/CDK2 complexes and likewise through the increased binding of p27 to cyclin E/CDK2 and inhibition of their kinase activity (Petrocelli, Slingerland, 2000). Defects in CDKN1A leads to defective p53 function, while defects in CDKN2A result in inadequate p16 function (Pavey et al., 2013). The loss of function in p53 and p16 leads to uncontrolled cell cycle regulation due to increased phosphorylation of CDK4/6 targets (Miller, Flaherty, 2014). Familial melanomas, which have germline mutations in p16INK4A, are associated with increased CDK4 activity (Miller, Flaherty, 2014). Dysregulation of CDKN2A, in conjunction with RAS and BRAF mutations, increases tumor cell invasion (Miller, Flaherty, 2014). CDK2 is regulated by MITF in melanocyte cells (Du et al., 2004). MITF-specific CKD2 regulation is linked to transcriptional regulation of SILV/PMEL17/GP100 activity, which leads to increased CDK2 activity. In contrast, silencing of MITF activity represses CDK2 activity, showing their co-regulation (Du et al., 2004).

Activation of p53 signaling after the UVR initiates cell cycle arrest, damage repair, autophagy of damaged proteins and apoptosis if the damage is severe. The UVR response simultaneously activates the AKT/mTOR signaling pathway, which acts oppositely to p53; their concurrent
activation can lead to cell senescence (Strozyk, Kulms, 2013). NRF2 controls the paracrine α-MSH secretion in keratinocytes, which in turn mediates UVB-induced DNA damage in melanocytes. NRF2 silencing reduces α-MSH secretion from keratinocytes and increases the melanocytes apoptosis by MAPK pathway activation (Jeayeng et al., 2017). In contrast, UVA mediates apoptosis in corneal epithelial cells via NRF2 induced ROS and activation of p53/Caspase3 signaling (Liu et al., 2016a).

2.3 HYALURONAN

2.3.1 Structure and function of hyaluronan

Hyaluronan (Fig. 3) is an unbranched sugar polymer which consists of repeating disaccharides of glucuronic acid (GlcUA) and N-acetylglucosamine (GlcNAc) (Weissmann et al., 1954). Hyaluronan belongs to the glycosaminoglycan family together with chondroitin sulphate, dermatan sulphate, keratan sulphate and heparin (heparan sulphate), but differs from them structurally and by its synthesis. Unlike other glycosaminoglycans, which are made as proteoglycans in the rough endoplasmic reticulum (rER) and Golgi apparatus, hyaluronan is synthesized as an unmodified linear polysaccharide \[-\beta(1,4)-\text{GlcUA}-\beta(1,3)-\text{GlcNAc}]-n by hyaluronan synthases (HAS) at the plasma membrane (Toole, 2004, Fraser, Laurent & Laurent, 1997). Hyaluronan synthases (1–3) are multipass transmembrane enzymes, which synthesize hyaluronan to the cell surface or into the extracellular matrix (Weigel, Hascall & Tammi, 1997). Crystallography analysis has revealed that hyaluronan can form helical structures of 2-fold polymers, stabilized by hydrogen bonds and hydrophobic interactions, in association with hyaluronan chains in β-sheet mesh-like structures. Nuclear magnetic resonance (NMR) and molecular dynamics (MD) methods have also revealed that the β1,3 linkage between the precursor sugars is more rigid, compared to the β1,4 linkage, which has the active site for hyaluronan degradation by hyaluronidases (Scott, Heatley, 2002, Scott, Heatley, 1999, Holmbeck, Petillo & Lerner, 1994).

Meyer and Palmer (1934) first discovered hyaluronan and described its mucous, because it is very hygroscopic and highly viscous in nature (Meyer et al., 1934). Later on, its chemical structure and unique properties were discovered. Regarding its water absorbing abilities, hyaluronan is extremely hydrodynamic and because of this property it has an important effect in tissue homeostasis and a biomechanical integrity. It has been known for a long time that hyaluronan has an important function in embryogenesis (Toole, 1972, Toole, 1990), extracellular matrix organization (Knudson, 1993, Goldberg, Toole, 1984), wound healing (Helin et al., 1972, Pinkus, Perry, 1953) and inflammation (Constable, Swann, 1972, Castor, Prince & Hazelton, 1966). Recently hyaluronan was found to modulate mammary gland morphogenesis (Tolg et al., 2017) and it is also involved in left-right asymmetry in gut rotation during development. Hyaluronan together with TSG-6 provides a matrix in the right side of the dorsal mesentery that makes the gut tilt to the left side. Loss of TSG-6 or inhibition of hyaluronan synthesis prevents the normal gut tilting and leads to strangulation of the gut vasculature (Sivakumar et al., 2017). Hyaluronan interacts with other proteoglycans and molecules to assemble the extracellular matrix. It regulates the plasticity of the matrix by facilitating cellular invasion during development, tissue remodeling and also in cancer progression (Toole, 2004, Tammi et al., 2008). Hyaluronan is present in the synovial fluid (Meyer, Smyth & Dawson, 1938) where it lubricates the joint and protects the cartilage. Hyaluronan resides in the cartilage around the lacunae of chondrocytes (Antonas, Fraser & Muirden, 1973) where it maintains cartilage integrity (Knudson, 1993). Half of the total body hyaluronan resides in the skin. In the skin, hyaluronan is produced mainly in the dermis by fibroblasts (Bertheim, Hellström, 1994) and in the epidermal spinous and basal layers by keratinocytes (Tammi et al., 1988).
2.3.2 Synthesis of hyaluronan

During hyaluronan synthesis, the growing chain protrudes into the extracellular space but it still remains bound to the synthase itself. Biochemically, hyaluronan synthases use the two sugar substrate precursors equally, uridine diphosphate- (UDP-) GlcNAc and UDP-GlcUA. These substrates are added to the reducing end of the hyaluronan chain by HAS and the terminal UDP is replaced by new UDP-sugar precursors in the presence of Mg\(^{2+}\) or Mn\(^{2+}\) (Prehm, 1984). Hyaluronan synthesis starts \textit{de novo} in HASes, which do not need a primer for the start of synthesis (Weigel, DeAngelis, 2007).

Precursor sugar UDP-GlcNAc is made from fructose-6-phosphate in the hexosamine biosynthesis pathway in the cytosol (Arreola et al., 2003) where it is used for hyaluronan synthesis (Rilla et al., 2013a, Prehm, 1984). Glutamine-fructose-6-phosphate aminotransferase 1 (GFAT) enzyme controls the rate-limiting step in the generation of D-glucosamine-6-phosphate from fructose-6-phosphate in the hexosamine pathway (Oikari et al., 2016). UDP-GlcUA on the other hand is synthesized in the glucuronic acid biosynthesis pathway (Hanover, Krause & Love, 2012) and made from glucose-6-phosphate through uridine diphosphate glucose intermediate (Li, 2010). The size of UDP-sugar precursor pools in the cytosol (Rilla et al., 2013a, Prehm, 1984) acts as a rate-limiting factor in hyaluronan synthesis. Precursors are affected by different metabolic conditions of the cells or the cell type itself (Tammi et al., 2011, Jokela et al., 2008a). UDP-GlcUA level can be reduced with 4 methylumbelliferone, 4-MU, (Kultti et al., 2009, Kakizaki et al., 2004), and UDP-GlcNAc level with mannose (Jokela et al., 2008a) or by inhibiting the GFAT1 enzyme (Oikari et al., 2016). The pool of UDP-GlcNAc can be increased with glucosamine (Jokela et al., 2008a), or by increasing GFAT enzyme activity either with cAMP-dependent phosphorylation (Chang et al., 2000) or overexpressing GFAT itself (Daniels et al., 1993). Glucosamine can directly phosphorylate glucose to glucosamine-6-phosphate and therefore bypass the first rate-limiting step of fructose-6-phosphate to glucosamine-6-phosphate (Marshall, Nadeau & Yamasaki, 2005), whereas GFAT enzyme overexpression can induce the synthesis of glucosamine-6-phosphate from its precursor fructose-6-phosphate (Schleicher, Weigert, 2000).

Interestingly UDP-GlcNAc is the most abundant nucleotide sugar in mammalian cells; its content in the brain, heart and liver tissue is 6–10 times higher than the content of UDP-GlcUA, whereas in liver it is only 1.8 times higher (Oikari, Venäläinen & Tammi, 2014). UDP-GlcNAc controls the O-GlcNAcylation of various proteins, such as HAS3, and its lysosomal degradation and shedding in extracellular vesicles (Deen et al., 2016). O-GlcNAcylated proteins are involved in many cellular processes such as in transcription (c-Myc, CREB, p53), cytoskeletal assembly (vimentin) and in signal transduction (PI3K, AKT) (Slawson, Copeland & Hart, 2010). UDP-GlcNAc content fluctuates in different metabolic stages of the cell. O-GlcNAcylation of proteins...
is increased in cellular stress responses, diabetes and in cancer. In cancer cells the metabolism is overactive and the sufficient pool of UDP-GlcNAc can cause an increase in hyaluronan synthesis and O-GlcNAc signaling. O-GlcNAcylation can also regulate β-catenin and E-cadherin trafficking in epithelial cells (Slawson, Copeland & Hart, 2010, Hart, Housley & Slawson, 2007). It is possible that the dysregulation in O-GlcNAcylation affects changes in E-cadherin and β-catenin in EMT, and this has an impact on melanomagenesis.

2.3.3 Hyaluronan synthases
The hyaluronan synthase family is found in vertebrates, in some bacteria and even in some viruses (Weigel, DeAngelis, 2007), with which they share sequence homologue (Weigel, Hascall & Tammi, 1997). In humans, hyaluronan is synthesized by three different hyaluronan synthases (HAS1–3); their sequence is homologous with each other, but they are situated in different chromosomes. HAS1 is located in chromosome 19 at q13.3–13.4, HAS2 in chromosome 8 at q24.12 and HAS3 in chromosome 16 at q22.1. Whereas HAS1 and HAS2 are found in one transcript, the HAS3 transcript has two variants, HAS3v1 and HAS3v2 (Weigel, Hascall & Tammi, 1997, Spicer et al., 1997, Spicer, Olson & McDonald, 1997). In B-cell lineage cancers, alternative transcripts for HAS1 have been characterized. These variants termed HAS1 Va, Vb and Vc, are formed by exon skipping or partial intron retention that occur independently or in conjunction. These variants influence the tumorigenicity of these B-cells (Ghosh, Kuppusamy & Pilarski, 2009). Using in silico and in vitro methods, the genomic structure of HAS genes has been analyzed. HAS1 contains 5 exons and the translation site starts 9 bp from 3’ end of exon 1. HAS2 contains 4 exons and the translation start site is located at nucleotide 1 of exon 2, whereas HAS3, which has two transcript variants, show similarity to HAS2. Both variants have 4 exons and exons 2 and 3 are similar with HAS2 and their translation site starts at nucleotide 1 at exon 2. The difference between HAS3v1 and HAS3v2 exists at exons 1 and 4 (Monslow et al., 2003).

Hyaluronan synthases are multipass transmembrane enzymes with the catalytic unit in the central domain (Fig. 4). The structural homologue between human HAS enzymes includes a cluster of 2–3 membrane-associated domains at the amino terminal as well as at the carboxyl end of the synthase (Weigel, Hascall & Tammi, 1997). The eukaryotic synthase itself comprises of seven membrane domains, including 4–6 transmembrane domains and 1–2 membrane associated domains, of the intracellular catalytic domain and of the intracellular N- and C-terminus. Two of the membrane domains are lacking from the bacterial HAS (Weigel, DeAngelis, 2007, Weigel, Hascall & Tammi, 1997).

![Figure 4. The suggested plasma membrane topology of the HAS family (modified from Weigel, Hascall & Tammi, 1997).](image-url)
Hyaluronan synthases are transported through the ER, Golgi and trans-Golgi network to the plasma membrane, where they achieve their active form (Rilla et al., 2005; Prehm, 1984). The typical chain length of produced hyaluronan is 1–10 MDa and can consist of 2,000–25,000 disaccharides, with length extending up to 25 µm (Weigel, DeAngelis, 2007; Toole, 2004). Even though HASes share sequence homology, their enzymatic properties differ. In vitro studies have revealed that in COS-1 and rat 3Y1 cells, HAS1 and HAS2 synthesize higher molecular mass hyaluronan (2x10^5–2x10^6) compared to HAS3 (10^5–10^6). The size distribution of hyaluronan synthesized by HAS1 or HAS3 is broader compared to hyaluronan synthesized by HAS2, which generated more of the high molecular weight (HMW) hyaluronan than the lower molecular weight (LMW) form. The determined kinetic values (K_m) differed between HASes; HAS3 being the most active at lower concentrations of UDP-sugar substrates and the activity of HAS2 was higher than that of HAS1 (Itano et al., 1999). Rilla et al. (2013) showed that HAS3 could produce hyaluronan even from low concentrations of UDP-sugar substrates, at which HAS1 is almost inactive. However, the UDP-sugar supply had little effect on the activity of HAS2 or HAS3 and it seemed that HAS2 is able to produce larger but more diffuse pericellular hyaluronan coat compared to HAS3 (Rilla et al., 2013a). The synthetic activity of HAS1 seems to be more dependent on induction by the glucose balance and cytokines than the activity of other HASes (Siiskonen et al., 2014) and it is central in cell stress, such as during inflammation (Siiskonen et al., 2015a). Recent studies have shown that Rab10 is a mediator for the clatrin-mediated endocytosis of HAS3 from the plasma membrane to degradation (Deen et al., 2014), but the intracellular mediators for the traffic of HASes from the Golgi to the plasma membrane still remain unsolved.

Developmental studies in mice showed that Has2 is expressed throughout embryogenesis in several tissues and remains the most significant hyaluronan synthase. In mice, Has1 is expressed until embryonic day (E) 7.5 and after E8.5 its expression disappears. Has3 expression appears at E10.5 localizing at first in the branchial arch and mandibula. The staining pattern of HASes using anti-Has antibodies correlated with the mRNA expression patterns in mouse embryos and hyaluronan was found in many tissues, although not all, throughout embryonic and fetal development (Törrönen et al., 2014; Tien, Spicer, 2005). Has2 knockout mice show embryonic lethality via severe cardiac and vascular abnormalities (Camenisch et al., 2000), whereas Has1 or Has3 knockout mice showed no obvious developmental defects. However, Has1 or Has3 were shown to be relevant in fibroblast wound closure in the dermis (Mack et al., 2012).

Overexpression of different HASes in in vitro cell cultures has shown that they have a distinctive effect in different cell types. Overexpression of HAS2 in breast cancer cells correlates with tumorigenesis and metastasis (Li et al., 2015) and suppresses tissue metalloproteinase inhibitor (TIMP-1) promoting invasion (Bernert, Porsch & Heldin, 2011). In renal proximal tubular cells, overexpression of HAS2 leads to increased cell migration (Selbi et al., 2006), whereas in glioma cells, it reduces cell proliferation when hyaluronidase expression was deficient (Enegd et al., 2002). HAS3 overexpression shows similar effects, promoting tumor growth in pancreatic cancer microenvironments (Kultti et al., 2014) and the growth of TSU prostate cancer cells (Liu et al., 2001). HAS3 overexpression also promotes microvillus-like cell protrusions in breast cancer MCF-7 cells (Kultti et al., 2006), disturbs the normal epithelial organization and causes disorientation of the mitotic spindle in Madin-Darby canine kidney cysts (Rilla et al., 2012) and promotes shedding of plasma membrane-derived microvesicles (Rilla et al., 2013b). Thus, while overexpression of HASes seems to have several unwanted effects in cells, cells clearly need normal HAS function and hyaluronan synthesis to function properly.

The inhibitor of hyaluronan synthesis, 4-MU, which reduces the precursor sugar UDP-GlcUA levels and decreases HAS2 and HAS3 expression (Kultti et al., 2009), restrains pulmonary hypertension associated with lung fibrosis by reducing hyaluronan synthesis (Collum et al., 2017). Furthermore, 4-MU prevents keloid scar formation in keratinocytes (Supp et al., 2014) is
able to sensitize myelogenous leukemia cells for doxorubicin treatment (Uchakina et al., 2016) and inhibit pancreatic cancer cell motility (Cheng et al., 2016). Treating breast cancer cells with 4-MU suppressed their tumorigenicity and bone metastasis in mouse models by suppressing Has2 expression and AKT phosphorylation (Urakawa et al., 2012). Knockdown of HAS2 sensitizes colorectal carcinoma and bone osteosarcoma cells for radiation therapy (Shen et al., 2014), whereas silencing HAS3 reduces subcutaneous colon cancer growth (Teng et al., 2011). These studies show the importance of HASes and hyaluronan synthesis for normal cellular function but also during situations where cellular homeostasis is disturbed.

2.3.4 Regulation of hyaluronan synthesis

Hyaluronan synthesis can be regulated either at the transcriptional or post-transcriptional level. Transcriptional regulators of HASes include cytokines, growth factors or hormone-type effectors. Factors released by the surrounding cells, such as IL-1β, interferon (IFN) γ, basic FGF (bFGF), epidermal growth factor (EGF), keratinocyte growth factor (KGF), PDGF, TGF-β, TNF-α, prostaglandins, corticosteroids or retinoids from the microenvironment, can either enhance or suppress HAS expression and hence hyaluronan synthesis (Tammi et al., 2011). Different HAS isoenzymes respond differently to various factors, but this also depends on the cell type. In normal human mesothelial (NHM) cells, PDGF-BB induces HAS2 expression, whereas TGF-β1 induced HAS1 expression (Jacobson et al., 2000). PDGF in different forms (-AA, -AB, -BB) seem to stimulate HAS2 in different cell types, such as PDGF-AB in arterial smooth muscle cells (Evanko et al., 2001), or in dermal fibroblasts where it upregulates both HAS2 and HAS1 transcription (Campo et al., 2007). In ovarian cancer cells EGF increases HAS2 transcription, slightly increases HAS3 transcription, whereas HAS1 is unaffected (Park, Ko & Kim, 2017). This same trend with EGF treatment is seen in lung adenocarcinoma cells (Chow, Tauler & Mulshine, 2010). In rat and human epidermal keratinocytes, Has2 was the main Has to respond to EGF treatment, although the expression of Has3 was also enhanced (Saavalainen et al., 2005, Pienimäki et al., 2001, Pasonen-Seppänen et al., 2003). In human periodontal ligament cells as well as in dental pulp cells, bFGF was found to upregulate the transcription of HAS1 and HAS2. These studies suggest a possible function for hyaluronan in the regeneration of periodontal tissue and in dental development (Shimabukuro et al., 2005a, Shimabukuro et al., 2005b).

The effect of TGF-β on HAS transcription is interesting. Studies show that it has dual effect, stimulating and reducing HAS transcription. In human fibroblast-like synoviocytes TGF-β1 stimulates HAS1 transcription, but suppress HAS3 transcription dose dependently (Stuhlmeier, Pollaschek, 2004a). Similarly, in human dermal fibroblasts, TGF-β1 has been shown to induce the expression of HAS1 and HAS2 but reduce HAS3 (Campo et al., 2007). In NHM cells, TGF-β1 decreased HAS2 expression and slightly increased HAS1 (Jacobson et al., 2000). This latter result was also shown in rat keratinocyte organotypic cultures, where TGF-β downregulated Has2 and Has3 expression, hyaluronan synthesis, keratinocytes’ proliferation and reduced the thickness of the epidermis, whereas EGF showed the opposite effects (Pasonen-Seppänen et al., 2003). Cytokines, such as IL-1β, IFN-γ and TNF-α also show transcriptional upregulation towards HASes. In dermal fibroblasts, these cytokines induce the transcription of all HASes (1–3) (Campo et al., 2006). Similar results were seen in human periodontal ligament cells, which do not express HAS1, where IL-1β and TNF-α induce HAS2 and HAS3 transcription (Ijuin et al., 2001). Finally, in rabbit synovial membrane cells, cytokines IL-1β, IFN-γ and TNF-α have been shown to induce rapid upregulation of HAS2 and HAS3 mRNA transcription (Tanimoto et al., 2001).

Prostaglandins have a transcriptional stimulating effect towards different HASes. Prostaglandin E2 activates HAS1 in human fibroblast-like synoviocytes (Stuhlmeier, 2007), whereas prostaglandin D2 upregulates HAS2 transcription in human orbital fibroblasts (Guo et al., 2010). In rat epidermal keratinocytes, Has2 has reported to be the primary responsive gene after all-trans retinoic acid (Saavalainen et al., 2005). The human keratinocyte HAS2 promoter area for transcription contains a cluster for retinoic acid responsive elements at -1230, which all-
trans-retinoic acid receptor binding is able to activate (Saavalainen et al., 2007). In human foreskin keratinocytes, retinoic acid, retinol or β-carotene (provitamin A) influence hyaluronan synthesis by inducing HAS3 transcription (Sayo, Sakai & Inoue, 2004) this is in contrast to rat epidermal keratinocytes. Glucocorticoids, such as hydrocortisone, inhibit HAS1 transcription by blocking p38 mitogen-activated protein kinase signaling (Stuhlmeier, Pollaschek, 2004b), whereas dexamethasone, another type of glucocorticoid, suppresses HAS2 transcription in human dermal fibroblasts, but does not influence HAS3 expression (Averbeck et al., 2010). Dexamethasone acts via cell-surface glucocorticoid-receptors leading to suppression of HAS2 transcription and shortening of its mRNA half-life (Zhang et al., 2000).

All of these different growth factors, cytokines and other type of factors are able to affect HASes either on the transcriptional level or post-transcriptionally; the potency usually depends (upregulation/downregulation) on the cell type. PMA (phorbol 12-myristate 13-acetate) also has a strong stimulating effect on HAS2 transcription in NHM cells (Jacobson et al., 2000), whereas in glioma cells, it has a strong effect on the activity of other hyaluronan synthases as well (Asplund et al., 1998).

Binding sites for transcription factors E2FF, MZF1, ZBPF and cAMP response element-binding protein (CREB) as well as E-BOX, EGRF, NF-κB and specificity protein (SP) have been identified in the proximal promoter regions of all HASes (Monslow et al., 2003). The initiation site for transcription of HAS2 has been determined to start at the 5’ terminus at exon 2, but exon 1 in the 5’ untranslated region (UTR) contains binding sites for SP1, NF-Y/CCAAT and NF-κB necessary for HAS2 transcriptional control (Monslow et al., 2004). SP1 and SP3 were found to be the principal mediators for efficient HAS2 transcription (Monslow et al., 2006).

Saavalainen et al. (2005) reported that the HAS2 gene has four responsive elements for Signal transduced and activator of transcription (STAT) binding and four for retinoic acid receptor (RAR) proteins in the promoter region, which respond to EGF and all-trans retinoic acid external stimuli (Saavalainen et al., 2005). A later study found three response elements sites for NF-κB and 11 for SP1 (Saavalainen et al., 2007). Jokela et al. (2011) reported that the response elements SP1 and Yin-Yang 1 (YY1) to the promoter corresponded to the level of precursor sugars (UDP-GlcNAc and UDP-GlcUA) and the regulation of HAS2 expression. SP1 and YY1 were found to be O-GlcNAcylated regulating their binding activity and hence HAS2 transcription (Jokela et al., 2011).

Similar to HAS2, the HAS1 promoter has an SP3 binding site, but it also contains a binding site for SMAD3. Transcription induced by TGF-β1 utilizes SMAD3, while the IL-1β induced signal involves SP3 (Chen et al., 2012).

The HAS3 promoter region is divided into two regions; -761 to -305 bp upstream from the TIS where the proximal promoter occurs, and -433 to -305 bp upstream from TIS where the region for the core promoter occurs. The promoter regions contain a classical GC, but not a TATA box, as well as binding sites for transcription factors such as C/EBP and NF-κB and SP1, which were found to be essential for promoter activity, similar to HAS2, as well as for core region basic promoter activity (Wang et al., 2015).

Post-translational modifications of HASes can determine their activity and the hyaluronan synthesis rate in cells. HASes can be activated by phosphorylation via protein kinase A, C and calcium-dependent protein kinase (Klewes, Prehm, 1994) as well as ERK at serine residue (Bourguignon, Gilad & Peyrollier, 2007). In contrast, phosphorylation by AMP kinase (AMPK) at Thr-110 reduces HAS activation (Vigetti et al., 2011). HAS2 activity is hypothesized to be regulated by N-glycosylation of a co-protein (Vigetti et al., 2009). HAS2 can also be monoubiquitinated at Lys-190 which regulates its enzymatic activity (Karousou et al., 2010). HAS can also form homodimers with itself or form heteromeric complexes, which can vary depending on the cell line. N-terminal and glycosyltransferase domain of the interacting HAS is suggested to be important for the formation of homo- and heteromers (Bart et al., 2015). Formation of complexes may modulate hyaluronan synthesis and enzyme activity. HAS3
homomeric complexes had the highest synthetic activity, whereas HAS1 homomeric complexes had the lowest (Karousou et al., 2010, Bart et al., 2015).

2.3.5 Hyaluronan degradation

Hyaluronidase enzymes have been classified by Meyer in 1971 in three classes based on their biochemical properties. Two groups are endo-β-N-acetyl-hexosaminidases, one in the vertebrate lineage and the other in the bacterial lineage. The third group is endo-β-glucuronidase, which are found in leeches and crustaceans. For hyaluronan degradation in mammals (EC 3.2.1.35), there are six isoforms; they belong to the group of carbohydrate-active enzymes, called the glycosidase family 56. The vertebrate hyaluronidases are also called endo-β-acetyl-hexosaminidases, because they catalyze hyaluronan at the β1,4 glycosidic linkages. Bacterial and fungal species contain similar kinds of hyaluronidases, but the properties and mechanisms differ from eukaryotic ones. Interestingly, hyaluronidases have little catalytic properties towards chondroitin and chondroitin sulphates, but their degradation occurs slowly and plays a minor role (Stern et al., 2007, Csoka, Frost & Stern, 2001, Stern, Jedrzejas, 2006).

Hyaluronan degradation occurs at the site of the β1,4 glycosidic linkage (Stern et al., 2007). The hyaluronidase structure displays a classical distorted (β/α)8 triose phosphatase isomerase (TIM) barrel fold, with a cleft at the wider end of the TIM barrel which holds the catalytic unit for hyaluronan binding and degradation. The mechanism of hyaluronan catalysis involves several steps, as resolved by Jedrzejas and Stern in 2005 (Jedrzejas, Stern, 2005). The process generates hyaluronan fragments that are further cleaved in the active site of the enzyme. The shortest hyaluronan fragments that can be cleaved by mammalian hyaluronidase enzymes are hexasaccharide and tetrasaccharide (Stern, 2008). Tetrasaccharides are further cleaved to monosaccharides by the lysosomal exoglycosidases β-glucuronidase and β-hexosaminidase (Stern, 2003).

The hyaluronan content in tissues is tightly regulated by their synthesis and degradation. During normal homeostasis and in healthy tissues, hyaluronan mainly exists in the high molecular weight form. The half-life of hyaluronan is two to three days or even weeks, depending on the tissue type, whereas in serum it is only two to five minutes. Hyaluronan degradation occurs locally in tissues and in lymph nodes, or can be transported to the kidneys, spleen and the liver sinusoidal endothelium for degradation via the lymphatic system and blood circulation; in the end, part of it is excreted in the urine (Tammi et al., 1991, Engström-Laurent, Hellstrom, 1990, Morales, Hascall, 1988, Fraser et al., 1981).

Human hyaluronidases include HYAL-1, HYAL-2, HYAL-3, HYAL-4, PH-20/Spam1 (sperm adhesion molecule 1) and pseudogene PHYAL1. Their genes are clustered in chromosome 3p21.3 (HYAL1–3) and chromosome 7r31.3 (HYAL-4, PH-20, Phyal1) (Csoka, Scherer & Stern, 1999). Hyaluronidases have unique tissue distribution, with HYAL-1 and -2 being the main forms in human somatic tissues. HYAL-2 seems to play a prominent impact in the degradation of hyaluronan. The degradation of hyaluronan starts in the extracellular space and at the cell surface (Stern et al., 2007, Culty, Nguyen & Underhill, 1992). Although both HYAL-1 and HYAL-2 have an acidic pH optimum, HYAL-2 is also found in the plasma membrane (in addition to lysosomes and endosomes); this is where its main activity has been suggested to be located (Andre et al., 2011). HYAL-2 is bound to the plasma membrane by a glycosylphosphatidylinositol- (GPI-) anchor, localized in cholesterol-rich lipid rafts (Müllegger, Lepperdinger, 2002). It colocalizes with CD44, which is needed for its activity (Bourguignon et al., 2004, Duterme et al., 2009, Hida et al., 2015). Shedding of CD44 can release HYAL-2 activity even to more distant locations of the ECM (Hida et al., 2015). HYAL-2 generates approximately 20 kDa-sized fragments of hyaluronan. These are transported to early endosomes and lysosomes where acid-active HYAL-1 continues the degradation to tetrasaccharides (Stern, Asari & Sugahara, 2006).

The promoter site of HYAL-2 does not contain the usual TATA box to start transcription; instead it contains a GC-rich area and several putative Sp1 sites, typical for genes whose
transcription can be started at multiple sites. In chondrocytes, HYAL-2 expression is not induced by various cytokines, suggesting its possible function as a housekeeping gene in these cells (Chow, Knudson, 2005). However, the possibility of selective usage of multiple starting sites emphasizes a distinct effect for HYAL-2 in different cell types, and at different stages of differentiation.

HYAL-1 appears to be secreted via the ER-Golgi pathway followed by endocytosis-mediated re-uptake with hyaluronan to the endosomes, and subsequent processing to the active form, present in lysosomes (Puissant et al., 2014, Boonen et al., 2014, McAtee et al., 2015). HYAL-1 participates in hyaluronan clearance from the circulation by degrading it in liver non-parenchymal cells, but it also participates in hyaluronan degradation in peripheral tissues, such as skin (Bourquiignon, Flamion, 2016). The HYAL-1 gene is unusually long, which generates two splice variants that bare different enzymatic activities (Lokeshwar et al., 2006). HYAL-1 deficiency leads to a lysosomal storage disease (mucopolysaccharidosis IX) affecting, for example, joints (Triggs-Raine et al., 1999). HYAL-1 expression has been reported to be downregulated in some cancers, such as ovarian and endometrial cancer (Nykkopp et al., 2010, Nykkopp et al., 2009, Nykkopp et al., 2015), or upregulated in other cancers, such as bladder (Hautmann et al., 2001), breast (Tan et al., 2011) and pancreatic (Kohi et al., 2016). The regulation of HYAL-1 expression is poorly understood, but the gene promoter contains estrogen response element and an SP1 binding site, through which estrogen can inhibit HYAL-1 expression (Edjekouane et al., 2016). HYAL-1 was also shown to increase prostate adenocarcinoma cell proliferation and motility via its catalytic activity. Externalization of HYAL-1 via the ER-Golgi pathway followed by endocytosis-mediated re-uptake with hyaluronan for biosynthetic precursors or re-secreted as a LMW form accelerated the tumor cell aggressive growth (McAtee et al., 2015).

The enzymatic properties of HYAL-3 and its function in hyaluronan catabolism still remain unsolved. Heterogeneous HYAL-3 gene mutations correlated with lymph node metastases in lung squamous cell carcinoma (Zhang et al., 2013). HYAL-4 has a function in the cleavage of chondroitin sulphates, but not in hyaluronan cleavage (Kaneiwa et al., 2010). HYAL-4 may play a role in resolving chondroitin sulphate proteoglycans in neural scar tissues (Tachi et al., 2015).

Human PH-20 associates with testes and is necessary for fertilization. It facilitates the penetration of sperm through the cumulus mass to the ovum found by Duran-Reynals in 1928 (Martin-Deleon, 2011). PH-20 localizes to the acrosomes of spermatids (lysosome-related structures) and functions as hyaluronidase as well as an adhesion protein (Lin et al., 1994). Blocking PH-20 in guinea pig serum with antibodies causes 100% infertility, which can be restored following removal of the antibody (Primakoff et al., 1997). Therefore, it may have a function in infertility or in contraception as a potential target for vaccines (Bandivdekar, 2014).

Recently Yamamoto et al. (2017) described a new hyaluronidase, TMEM2, which is a mammalian homolog of the zebrafish transmembrane protein 2. It is localized on the cell surface as a type II transmembrane protein, and could degrade HMW hyaluronan to 5 kDa fragments, but do not degrade chondroitin sulphate or dermatan sulphate. The optimum pH for TMEM2 is between 6–7 and it is Ca2+-dependent. It is widely expressed in different cell types, but its effect in mammals remains to be resolved (Yamamoto+Tobisawa, 2017). KIAA1199; also known as CEMIB/HYBID hyaluronan-binding protein, is involved in hyaluronan binding and also has a function in its catabolism. KIAA1199 is located both in the cytoplasm and on the plasma membrane; it catalyzes hyaluronan in an endo-β-N-acetylglucosaminidase-dependent manner via the clathrin-coated pit pathway (Yoshida et al., 2013) and it controls hyaluronan depolymerization in endochondral ossification. KIAA1199-deficient mice showed hyaluronan accumulation, which resulted in reduced chondrocyte growth and adult bone length (Shimoda et al., 2017). Its expression is also increased in several cancers such as gastric and colorectal tumors (Chivu Economescu et al., 2010, LaPointe et al., 2012) and oral squamous cell carcinoma (Chanthammachat et al., 2013). KIAA1199 has also been shown to be involved in cellular
motility, invasion, proliferation and adhesion (Jia et al., 2017, Zhang et al., 2017, Jami et al., 2014, Evensen et al., 2013).

2.3.6 Hyaluronan binding proteins

Hyaluronan binding proteins, also called hyaladherins, exhibit differences in their tissue expression, cellular localization and regulation, as well as their affinity and specificity. They can be divided into cell-surface proteins, located in the plasma membrane, and matrix hyaluronan-binding proteins, which reside in the ECM, or by a shared structural domain called Link module and non-Link module hyaladherins (Day, Prestwich, 2002, Knudson, Knudson, 1993). The Link module contains 100 amino acid domain, that is involved in ligand binding in these receptors, which include; aggrecan found in the cartilage, brevican in neurons, hyaluronectin in soft connective tissue and nervous tissue, neurocan synthesized by fibroblasts (Knudson, Knudson, 1993), brain-specific link protein (BRAL1) (Hirakawa et al., 2000), cell surface receptors such as cluster of differentiation 44 (CD44) (D’Souza, Datta, 1986), tumor necrosis factor-stimulated gene 6 (TSG-6) (Lee, Wisniewski & Vilcek, 1992), Layilin (Bono et al., 2001) as well as lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) (Banerji et al., 1999, Banerji et al., 2016). Hyaluronan receptor for endocytosis (HARE), also known as Stabilin-2, is a type I membrane protein containing extracellular domain with a Link module and a transmembrane domain (Zhou et al., 2002).

Hyaluronan-binding proteins that are not included in the Link module family are Inter-α-inhibitor (IαI) (Huang, Yoneda & Kimata, 1993), CD38 (Nishina et al., 1994), plasma hyaluronan-binding protein, PHBP or HABP2 (Choi-Miura et al., 1996), interphotoreceptor matrix receptors sialoprotein (SPACR) (Acharya et al., 1998) and sialoproteoglycan SPACRCAN (Acharya et al., 2000). Intracellular proteins without Link module are P-32 or HABP1 (Deb, Datta, 1996), cell cycle control protein homologue CDC37 (Grammatikakis et al., 1995) and intracellular hyaluronan binding protein (IHAB4) (Huang et al., 2000). Receptor for hyaluronan-mediated motility (RHAMM) (Hardwick et al., 1992) is a largely cytoplasmic protein, but is also apparent on the cell plasma membrane (Assmann et al., 1998, Schwertfeger et al., 2015). RHAMM binds hyaluronan through a distinct domain that contains BX-B motifs (B is basic amino acid residue and X are non-acidic residues) (Yang, Zhang & Turley, 1993). Toll-like receptors (TLR), which are used normally in host defense initiation by the innate immune system can relay hyaluronan fragment-mediated cytokine production and signaling (Schwertfeger et al., 2015). Toll-like receptors, such as TLR-4, at the cell surface are activated by low molecular weight hyaluronan (Termeer et al., 2002).

Hyaluronan receptor CD44

CD44 is the most studied hyaluronan receptor. CD44 is a type I transmembrane glycoprotein, first characterized as a cell-surface molecule involved in organ-specific homing of lymphocytes (Gallatin, Weissman & Butcher, 1983). CD44 consists of an extracellular globular N terminal domain, a stalk domain that connects the N terminus and C terminus and a single-pass transmembrane and a cytoplasmic domain. The N terminal domain binds hyaluronan (Guvench, 2015). The CD44 gene contains 20 exons from which first 5 and the last 5 are constant and 10 exons between these can be alternatively spliced. This gives multiple CD44 isoforms ranging from 85 kDa to 230 kDa in size. The standard form of CD44 is an 85 kDa protein (Day, Prestwich, 2002, Naor, Sionov & Ish-Shalom, 1997, Goldstein et al., 1989). All isoforms contain about 170 amino acids in sequence in the amino terminal, which contains 6 cysteine residues that are considered to form 3 disulfide bonds and 5 possible N-linked glycosylation sites. In this region, about 100 amino acids long sequence shares homologue to other hyaluronan binding proteins, such as the Link-module proteins (Lesley et al., 2000).

Post-translational modifications of CD44 include phosphorylation of serines in the cytoplasmic domain, palmitoylation at C286 in the transmembrane domain and at C295 in the cytoplasmic domain, N- and O-linked glycosylation and the addition of glycosaminoglycan side
chains such as heparan sulphate or chondroitin sulphate (Thankamony, Knudson, 2006, Lesley et al., 1997, Naor, Sionov & Ish-Shalom, 1997, Liu, Sy, 1996, Pure et al., 1995). Chondroitin and heparan sulphate associate with CD44 isoforms v3−10 and v3,8−10. CD44 isoforms v10, v8−10, v7−10 and v6−10 are shown to lack these glycosaminoglycans, but are extensively O-glycosylated (Jackson et al., 1995). CD44 with variable exon v3 shows weaker hyaluronan binding capacity compared to the standard form of CD44 (Jackson et al., 1995, Bennett et al., 1995). CD44v3−10, called epican, is most abundantly expressed in the epidermis, especially by keratinocytes and it has a heparan sulphate glycosylation (Kugelman et al., 1992, Tuhkanen, Tammi & Tammi, 1997).

The extracellular N terminal domain of CD44 also interacts with other ECM molecules than hyaluronan, such as fibronectin, laminin, collagen and chondroitin sulphate. The intracellular domain of CD44 associates with the actin cytoskeleton via binding to ezrin, moesin and radixin (Nagano, Saya, 2004, Lesley et al., 1997, Naor, Sionov & Ish-Shalom, 1997). IL-1β is also shown to foster CD44-ezrin association by decreasing phosphorylation of CD44 at Ser-325 and promoting its homomerization in epidermal keratinocytes (Jokela et al., 2015). Amino acid residues at the Link module are shown to bind hyaluronan (Banerji et al., 2007). X-ray crystallography revealed that the Arg-41 residue in the loop structure of CD44 has the highest affinity for hyaluronan. Using computational techniques, Vuorio et al. (2017) showed that hyaluronan-CD44 interaction occurs via three distinct topographical modes; crystallography mode, upright mode and parallel mode. The parallel binding mode is the most frequent, where the hyaluronan chain lies on the top of the Arg-41 residue (Vuorio, Vattulainen & Martinez-Seara, 2017).

CD44 is essential for cell-cell and cell-matrix interactions. Likewise, it is involved in many processes such as T cell activation, leukocyte migration in inflammation as well as tumor metastasis, which are often associated with proteolytic cleavage of CD44 resulting in extracellular and intracellular parts of the protein (Heldin et al., 2008). Proteolytic cleavage of CD44 regulates its function. Soluble form of CD44 is responsible for ECM-CD44 interactions in migrating cells in a hyaluronan-rich microenvironment and acts as a competitive inhibitor for hyaluronan-CD44 binding. The amount of soluble CD44 was found to correlate with tumor burden and metastasis (Senbanjo, Chellaiah, 2017, Okamoto et al., 1999, Trapasso et al., 2016). The formation of soluble CD44 is generated by changes in the extracellular Ca²⁺ influx and mediated by MMPs, PKC activation or the activation of RAC and RAS oncoproteins. In particular, RAS mutations occur in invasive and metastatic tumors that show enhanced CD44 cleavage accompanied by an increased CD44-mediated migration (Kawamoto et al., 2000). Other mediators that can activate CD44 ectodomain cleavage are metalloproteinases ADAM10, which is induced by Ca²⁺ influx, and ADAM17, which is induced by PKC activation (Nagano et al., 2004). In invasive tumor cells, membrane type 1 MMP (MT1-MMP) is shown to localize in lamellipodia and to bind to the actin cytoskeleton via CD44 binding (Mori et al., 2002).

Growth factors and their receptors associate tightly to CD44 signaling and expression. CD44 can form a complex with the EGFR which causes phosphorylation and activates downstream signaling cascades such as RAS, RHOA and PI3K pathways and controls cell growth and survival (Wang, Bourguignon, 2011). TGF-β regulates CD44v6 expression through ERK signaling in pulmonary fibroblasts (Ghatak et al., 2017b) as well as NOX4/ROS signaling in myofibroblast differentiation in idiopathic pulmonary fibrosis (Ghatak et al., 2017a). CD44-TGF-β interactions can even induce EMT through upregulation of AKT/GSK3β/β-catenin pathway activity in hepatocellular carcinoma cells (Park et al., 2016). In pancreatic cancers, inhibition of CD44v6 with v6 peptides reduces xenograft growth and metastasis (Matzke-Ogi et al., 2016). CD44 can interact with PDGF-BB receptor β (PDGFRβ) and externally added hyaluronan inhibits PDGF-BB mediated CD44-PDGFRβ activation reducing cell migration. In dermal fibroblasts, PDGFRβ also interacts with TGF-β type I receptor (TβRI) and together they interact with CD44. Silencing of CD44 over-activates SMAD2 signaling via PDGFRβ-TβRI and prolongs PDGF-BB phosphorylation and ERK1/2 signaling. Signaling of PDGF-BB and TGF-β are
increased with EMT changes, suggesting a controlling function for CD44 in PDGFRβ-TβRI activated EMT alterations (Porsch et al., 2014, Li, Heldin & Heldin, 2006).

Melanoma cells show a constitutive expression of functionally different N- and O-glycosylations at amino acid sites of 180, 190, 231 and 258 respectively, in the standard form of CD44. These were found both in vitro as well as in vivo from melanoma cells obtained from primary and metastatic lesions (Gasbarri et al., 2003), and also melanoma cells, were shown to express all CD44 mRNA variants (Raso-Barnett et al., 2013). CD44v8–v10 induces melanoma cell extravasation due to loss of endothelial junction and activation of VE-cadherin (Zhang et al., 2014). CD44v6 correlates with the frequency of brain metastases when expressed in primary melanoma tumors (Marzese et al., 2015) and isoform switching from CD44std to CD44v9 in gallbladder cancer leads to increased tumorigenesis (Miwa et al., 2017). High expression of CD44 in the cancer microenvironment by cancer-associated fibroblasts (CAFs) can sustain the stemness of cancer cells and produce drug resistance (Kinugasa, Matsui & Takakura, 2014).

The molecular weight of hyaluronan can influence its interaction with CD44 and the signals it causes. The interaction of 500 kDa hyaluronan (intermediate MW) with CD44 has been shown to enhance neutrophil phagocytosis and IL-8 secretion via the activation of p38 and ERK1/2 signaling (Lu et al., 2017). High molecular weight hyaluronan downregulates MMP-1 and MMP-3 expression via CD44 binding (Wu et al., 2017) and downregulates TNF-α induced MMP-13 expression in chondrocytes (Furuta et al., 2017), whereas ultra-LMW (<10kDa) hyaluronan triggers cell necrosis of leukemia cells with high CD44 expression (Kasai et al., 2017).

**Hyaluronan receptors RHAMM and TLR**

RHAMM, receptor for hyaluronan-mediated motility, expression is increased in response to injury, but it is not detected in most homeostatic tissues. RHAMM locates in the cytoplasm in the mitotic spindle during cell division and on the cell surface, where its expression is tightly regulated and mostly involved in cell locomotion (Schwertfeger et al., 2015). In vascular smooth muscle cells hyaluronan induces RHAMM-mediated PI3K-RAC activation and cell migration (Gouëffic et al., 2006), whereas in pleural mesothelioma RHAMM-induced migration signals through the YAP1/TAZ axis (Shigeeda et al., 2017). RHAMM can mediate constractive artery wall remodeling (Ma et al., 2014b) and stimulate angiogenesis in wound healing via hyaluronan oligosaccharides (Gao et al., 2008). RHAMM-negative fibroblasts also show defective skin wound repair due to insufficient CD44-ERK1/2 activation (Tolg et al., 2006). RHAMM induces PI3K and MAPK signaling pathways in human choriocarcinoma cells after LMW hyaluronan activation (Mascaro et al., 2017). RHAMM upregulation is associated with metastasis and poor prognosis in many cancers such as non-small cell lung cancer (Wang et al., 2016), hepatocellular carcinoma (He et al., 2015) and colorectal carcinoma (Lugli et al., 2006). In melanoma, its function is still unclear. However, DNA vaccine for immunotherapy based on Xenopus RHAMM used in melanoma mouse models showed inhibition of angiogenesis, tumor growth and lung metastasis (Yang et al., 2010b), pointing towards a new subject for further studies.

Toll-like receptors, which are used normally in host defense initiation by the innate immune system, can mediate hyaluronan fragment-mediated cytokine production and signaling (Schwertfeger et al., 2015). The receptors that have shown to activate by hyaluronan fragments are TLR-2 and TLR-4. After activation, these receptors induce proinflammatory reactions (Schwertfeger et al., 2015, Jiang, Liang & Noble, 2011). The effect of TLR in melanoma has not been investigated, but TLR is associated with poor prognosis in non-small cell lung carcinoma (Bauer et al., 2017). TLR-4 mediated NF-κB signaling in ovarian carcinoma poses drug resistance (Sun et al., 2018) as well as high expression of TLR-2 which is linked to B-cell chronic lymphocytic leukemia (Rybka et al., 2016). However, the role of hyaluronan was not investigated in these studies, so the question still remains open whether hyaluronan is involved in TLR-induced signaling in these cancers.
2.3.7 Molecular weight of hyaluronan and its fragments

Hyaluronan metabolism is tightly regulated, which is crucial for tissue homeostasis, wound healing, regeneration and repair (Kavasi et al., 2017, Tammi, Day & Turley, 2002, Longaker et al., 1991) and the size of hyaluronan determines its impact on cells or the surrounding microenvironment (Marcellin, Steen & Nielsen, 2014). Hyaluronan can be produced as a HMW hyaluronan and later degraded to smaller sizes by hyaluronidases (Roden et al., 1989). In normal physiological situations hyaluronan synthases synthesize HMW hyaluronan (Laurent, 1987). It is involved in many biological functions such as in supporting the ECM and tissue integrity, in ovulation, promoting cell quiescence and in expanding intravascular volume in shock. High molecular weight hyaluronan accumulation in tissues promotes longevity and cancer resistance in naked mole rats, which was due to the unique sequence of HAS2 (Tian et al., 2013). But the motive that drives hyaluronan polymer length in vivo is still uncertain.

Hyaluronan depolymerization to low molecular weight can be generated enzymatically by hyaluronidases and non-enzymatically by ROS. ROS fragment hyaluronan randomly from their internal glycoside linkages (Deguine et al., 1997). In contrast to HMW hyaluronan in normal, healthy tissues, LMW hyaluronan or hyaluronan fragments (<250 kDa) are thought to be a sign of a tissue injury (Noble, 2002). Low molecular weight hyaluronan has been shown to induce upregulation of inflammatory cytokines and chemokines (Vistjnova et al., 2014, Bourguignon et al., 2011, Ochoa et al., 2011) and small oligosaccharides (<20 kDa) have been shown to enhance angiogenesis, inflammation and stimulate the immune system by activating dendritic cells (Termeer et al., 2000, Termeer et al., 2002). Hyaluronan fragments up to 20 disaccharides can be angiogenic (Cui et al., 2009, West et al., 1985), while hyaluronan of 2−45 kDa (low and intermediate size) can induce macrophages to produce chemokines, such as MIP-1α (macrophage-inflammatory protein), MIP-1β, RANTES and IL-8 (McKee et al., 1996), and TGF-β from eosinophils (Ohkawara et al., 2000). The upregulation of these chemokines and growth factors induce endothelial cell migration (Sattar et al., 1994) and dendritic cells maturation (Termeer et al., 2000).

The effects of hyaluronan fragments in tissue responses are mediated through receptors such as CD44, RHAMM or TLR-2 and -4 (Schwertfeger et al., 2015). Hyaluronan oligosaccharides can induce inflammatory chemokines or MMP expression by activating NF-κB signaling (Fieber et al., 2004) or by inducing TLR-2 and TLR-4 mediated signaling which activates NF-κB nuclear localization and results in TNF-α and IL-1β secretion (Campo et al., 2013). In melanoma cells, hyaluronan fragments can upregulate IL-8 and MMP-2 by activating TLR-4-specific signaling which leads to NF-κB nuclear localization (Voelcker et al., 2008). Hyaluronan fragments of 4−16 oligosaccharides also induce the maturation of dendritic cells via autocrine signaling of secreted TNF-α, which is able to stimulate T-cells (Termeer et al., 2000). The maturation of the dendritic cells occurs via CD44 and RHAMM independent and TLR-4 dependent mechanisms (Termeer et al., 2002).

Oligosaccharides have also been shown to promote fibroblasts migration during wound closure and to recruit M1- and M2-type macrophages (Tolg, Telmer & Turley, 2014). They have also been shown to induce the differentiation of keratinocytes by increasing the expression of integrins α6 and β1 in the basal layer of the epidermis (Choi et al., 2012). Hyaluronan oligosaccharides (6-mer) may participate also in neuroinflammatory reactions in neuroblastoma cells. External hyaluronan 6-mer induced neuroinflammation via TLR-2, TLR-4 and CD44 receptors, which mediated NF-κB activation and α-synuclein production; this was associated with Lewy bodies in the degenerated neurons of Parkinson's disease (Scuruchi et al., 2016).

ROS can act in skin inflammation caused by contact allergens, generating hyaluronan fragments that activate MAPK-p38 and the NF-κB signaling pathway resulting in the induction of hyaluronidase activity and IL-6 production (Esser et al., 2012). In chronic airway inflammatory disease models, ROS-induced hyaluronan fragments binding to CD44 induced EGF release and CD44/EGFR interactions leading to increased mucin 5AC expression and mucus secretion in vitro (Yu et al., 2011b). The anti-oxidant N-acetylcysteine (NAC) was able to...
inhibit hyaluronan-fragment induced TNF-α expression and NF-κB activation in alveolar macrophages (Eberlein et al., 2008). These studies clearly demonstrate the effect of ROS in hyaluronan fragmentation and its involvement in NF-κB-mediated transcription of inflammatory cytokines. Another significant producer of ROS is UVR, which can activate many signaling pathways including MAPK, PI3K/AKT and transcription factors NF-κB and STAT3 (Xu, Fisher, 2005; Bubici et al., 2006). Hasova et al. (2011) found externally added hyaluronan (970 kDa) reduced the inflammatory effect of UV irradiation and decreased the secretion inflammatory cytokine IL-6 and chemokine IL-8 in UV-irradiated human keratinocytes (Hašová et al., 2011). In the epidermis, UVR was also found to increase hyaluronan catabolism by elevating the expression of HYAL-1 in keratinocytes (Kurdykowski et al., 2011), whereas mouse skin exposure to UVB showed decreased hyaluronan content in the dermis due to downregulation of all the Hases (Dai et al., 2007). Whole tissue analysis revealed from photoaged skin that photoaging induces a downregulation of HAS1 and an increase in all hyaluronidases, as well as increase in hyaluronan content. The study concluded that while hyaluronan content increases during photoaging, it is towards LMW hyaluronan (300 kDa) and possibly due to activation of hyaluronidases, which could have a further inflammatory function (Tzellos et al., 2009).

2.3.8 Hyaluronan in cell cycle regulation

The effect of hyaluronan in cell cycle regulation and control has been studied extensively. It is shown that cyclin D3 regulates hyaluronan synthesis activity in rat mesangial cells. In these growth-arrested cells, serum or glucose stimulation increases cyclin D3 activity together with colocalization of CDK4 and C/EBPα, which is responsible for hyaluronan rich extracellular matrix formation (Ren, Hascall & Wang, 2009). Kaul et al. (2012) showed that overexpression of hyaluronan binding protein HABP1/P-32 in HepG2 cells upregulated the expression of HAS2 leading to increased hyaluronan synthesis, and the expression of cyclin D1, which enhanced cell proliferation (Kaul et al., 2012). Extracellularly added hyaluronan has been shown to cause activation of cyclin D1 via Wnt/β-catenin signaling leading to increased proliferation in mesenchymal cells (Liu et al., 2016b). In another study, it was revealed that HMW hyaluronan CD44 interaction inhibited cyclin D1 expression via RAC-dependent signaling, whereas LMW hyaluronan binding to CD44 increased cyclin D1 via the activation of ERK signaling (Kothapalli et al., 2008). Another example of hyaluronan’s effect in cell cycle regulation is the naked mole rats that express very high molecular weight hyaluronan which gives them cancer resistance (Tian et al., 2013). The naked mole rats express alternative transcription in INK4 locus, which produces an isoform of p16^INK4a and p15^INK4b termed pALT^INK4ab (Tian et al., 2015). This isoform is expressed during early contact inhibition and during a variety of stresses such as UV-exposure induced senescence; it also promotes stronger cell cycle arrest compared to p16^INK4a or p15^INK4b. Hyaluronidase treatment prior to early contact inhibition can reduce pALT^INK4ab expression and induce rapid cell growth (Tian et al., 2015).

2.4 TUMOR MICROENVIRONMENT

2.4.1 Components of tumor microenvironment

For melanoma dissemination, malignant cells need support from the surrounding cells - it is not enough that malignant cells themselves form neoplastic lesions. In the early phase, neoplastic cells are restricted from the surrounding tissue by the basement membrane and at this point, the local tumor is called carcinoma **in situ**. The interaction between the cancer cells and the surrounding microenvironment is the determinant of cancer metastasis (Kalluri, Zeisberg, 2006). In addition to cancer cells, the tumor microenvironment (TME) consists of several other
cell types such as fibroblasts, endothelial cells and immune cells and the ECM (Wang et al., 2017a).

**Tumor ECM**
The extracellular matrix in the TME consists of a dynamic network of different macromolecules and gives the tissue its unique composition. The main macromolecules in the ECM are collagens (I and III), elastin, hyaluronan and proteoglycans such as decorin and versican or fibronectin (Sainio, Järveläinen, 2014). Collagens in the ECM provide strength to the tissue, regulate cell adhesion, support chemotaxis and migration and regulate tissue development (Rozario, DeSimone, 2010), whereas elastic fibers provide the elasticity and resilience to the tissue (Wise, Weiss, 2009). Fibronectin mediates cell attachment and directs the organization of interstitial ECM and is involved in cell migration and acts an extracellular mechanoregulator (Rozario, DeSimone, 2010, Smith et al., 2007). Hyaluronan in the ECM fills the extracellular space within the tissue microenvironment and forms hydrated gel-like “goo” that holds the connective tissue together (Toole, 2004). It forms a basis for specific interactions with other ECM macromolecules and interacts with cell surface receptors that transduce intracellular signaling and influence cell behavior (Itano, Zhuo & Kimata, 2008). These unique properties of hyaluronan make it special in cancer progression. Hyaluronan surrounding tumors can reduce drug delivery by masking the cells from efficient treatment (Provenzano et al., 2012). An imbalance in hyaluronan turnover in cell glycocalyx induces aberrant hyaluronan content, which actsuates malignant behavior in many cellular microenvironments, such as in mammary tumorigenesis (Itano, Zhuo & Kimata, 2008). Increased hyaluronan production weakens the cells contact inhibition and promotes migration in rat fibroblasts (Itano et al., 2002), whereas in naked mole rats it increases early contact inhibition (Tian et al., 2013, Tian et al., 2015). Hyaluronan also governs the crosstalk between tumor and stromal cells, but the full mechanism is still unknown. There are many possibilities, and it is likely the increased accumulation of hyaluronan influences stromal turgidity, disrupts cell-cell junctions, promotes stromal cell motility or induces intracellular signaling via its cell surface receptors, such as CD44 or RHAMM (Itano, Zhuo & Kimata, 2008).

Integrins in the ECM are adhesion molecules, which connect the ECM to the cellular cytoskeleton and transmit intracellular signaling, acting on the pathways involved in cell proliferation and migration, but also in cell survival and invasion (Bauer, Hein & Bosserhoff, 2005). Integrins are not classical signaling receptors, nor do they possess enzymatic activities; instead by their ability to connect with growth factor receptors present in the adhesion sites, integrins can modulate growth factor signaling (Munger, Sheppard, 2011). Fibronectin binding to integrins such as αvβ3 mediates cell adhesion, which is involved in the EMT process (Jia et al., 2010). Integrin αvβ3 also associates with active MMP-2 on the surface of invasive cells and regulates cell migration and ECM degradation by cleaving fibronectin (Jiao et al., 2012, Brooks et al., 1996). Increased αvβ3 integrin expression is linked to the loss of E-cadherin expression in transforming melanocytes, and this is controlled by the PTEN/PI3K pathway, which transforms radically growing cells to the vertical growth phase in melanoma (Albelda et al., 1990).

Besides space filling macromolecules, secreted proteins are involved in ECM modulation. These include MMPs, disintegrin and metalloproteinases (ADAMs), ADAM with thrombospondin motifs (ADAMTS), lysyl oxidase enzymes (LOX), thrombospondins 1 and 2, tenascin C, and osteopontin; all of which modify and remodel the ECM (Sainio, Järveläinen, 2014). MMPs modulate the ECM as a result of external stimuli. MMP secretion is active in invasive cells where the degradation of collagen type I and IV is increased (Blackburn et al., 2007). Increased expression of MMP-1, MMP-2 and MMP-9 correlates with poor prognosis, low survival rate and increased metastasis in melanoma (Botti et al., 2013). Osteopontin is mainly present in bones, but its expression is increased in metastatic melanomas compared to normal nevi and its high expression level correlates to low PTEN level (Packer et al., 2006, Zhou et al., 2005). Due to alternative splicing, tenascin C exists in various isoforms and is expressed in normal and neoplastic tissues. In melanoma, tenascin C has an effect in cell invasion and it
mediates integrin-mediated cell adhesion and re-organization of the ECM (Botti et al., 2013, Sriramarao, Bourdon, 1996).

**Growth factors in tumor microenvironment**
The crosstalk between different cells mediated by growth factors, cytokines and chemokines via paracrine and autocrine signaling, is a just one of many driving forces in tumorigenesis. Paracrine signaling differs between cell types and the origin of tumor. This paracrine signaling is bi-directional, both cancer and stromal cells secrete growth factors and cytokines. Fibroblasts localized in the stromal tissue supply the paracrine growth factors to the epithelial cells and maintain tissue homeostasis (Lee, Herlyn, 2007). Growth factors such as bFGF, VEGF, PDGF and EGFR ligands, together with TGF-β and interleukins (IL-1), modulate the TME and favor tumor growth (Mueller, Fusenig, 2004). These factors promote vascularization and stimulate inflammation, which in turn activate stromal fibroblasts. Production of proteolytic enzymes, such as MMPs, remodels the ECM which favors migration and invasion (Hsu, Meier & Herlyn, 2002, Ruiter et al., 2002). Growth factors can activate signaling pathways such as MAPK and PI3K, phospholipase C-γ (PLC-γ) or activate transcription factors such as STAT or SMAD. Growth factors are able to enrich the pool of cells susceptible for mutations (Witsch, Sela & Yarden, 2010). MMPs (Ziani et al., 2017) and cytokine IL-6 secreted by cancer-associated fibroblasts (CAFs) combined with melanoma cells secreting IL-8, induce melanoma cell invasion (Jobe et al., 2016). In addition, Notch1 signaling activity in CAFs determine their phenotype. Low Notch1 activity in cancer-associated fibroblasts promotes melanoma invasion and active tumor stroma (Shao et al., 2015). Heterogeneous population of cells in the tumor stroma can even promote drug resistance in melanoma cells. The stromal cell population secreting HGF activates MET-receptor in melanoma cells, which lead to sustained activation of ERK and AKT signaling and BRAF inhibitor resistance (Straussman et al., 2012).

The pattern of secreted growth factors is changed over time during melanoma progression. Most of the primary tumors express IL-15, insulin-like growth factor 1 (IGF) and bFGF, while their expressions are significantly reduced in metastatic melanoma tumors (Elias, Hasskamp & Sharma, 2010). Similarly, primary melanoma cells show intense TGF-β staining, which is declined in metastatic tumors. Instead, EGF is expressed in both primary and metastatic tumors almost equally intensely (Elias, Hasskamp & Sharma, 2010). Stromal fibroblasts secrete IGF-1, which is able to induce melanoma cells survival via MAPK-ERK1/2 pathway (Satyamoorthy et al., 2001). TGF-β is interesting, since it has both suppressive and promoting functions. In primary melanocytes and keratinocytes, it acts as growth-suppressor, whereas in metastatic melanoma, it induces proliferation and differentiation (Lee, Herlyn, 2007). TGF-β also induces stromal fibroblasts to produce ECM proteins such as fibronectin, tenascin C and collagens I, III, IV, VI, XV and XVIII, which in turn increases the cancer cells metastatic potential (Curran, Keely, 2013, Berking et al., 2001).

**Tumor stroma - immune cells**
The surrounding microenvironment of tumors, so called tumor stroma, consists of mesenchymal cells such as fibroblasts, neuroendocrine cells, immune and inflammatory cells, adipose cells and blood and lymph vessel network. Each of them has a unique function in modulating the TME (Wang et al., 2017a). Fibroblasts maintain the homeostasis in the ECM by regulating the synthesis of ECM molecules and the secretion of the degrading proteases such as the MMPs. In normal situation, the surrounding stroma has a minimal number of fibroblasts embedded in the surrounding physiological ECM, while in reactive stroma (desmoplastic), or cancer stroma their number is increased combined with escalated capillary network density and increased production of type I collagen, fibrin, hyaluronan and the infiltration of inflammatory cells (Augsten, 2014, Itano, Zhuo & Kimata, 2008, Kalluri, Zeisberg, 2006, Mueller, Fusenig, 2004). In some type of cancers, such as breast cancer, increased production of ECM hyaluronan by stromal fibroblasts, correlates with the metastatic stage of the tumor and favors the cancer
progression (Auvinen et al., 2000). In addition, the production of matrix remodeling enzymes such as neuron glial antigen (NG2) and MMP-3 induces the changes in the ECM composition (Spaeth et al., 2009, Sugimoto et al., 2006), which favor tumor progression.

Infiltration of lymphocytes, macrophages, mast cells, and neutrophils affect tumor progression and development (Botti et al., 2013). Immune cells of the stroma are divided to innate and adaptive immune cells, myeloid and lymphoid lineages. Lymphocyte cells in the stroma include T-cells, B-cells and natural killer cells (Yang, Lin, 2017). Tumor infiltrating lymphocytes (TIL) such as regulatory T-cells, cytotoxic T-cells, helper T-cells and regulatory B-cells recruit other immune cells, especially tumor-associated macrophages to the tumor site (Kitamura, Qian & Pollard, 2015, Botti et al., 2013). Myeloid-derived suppressors cells (MDSC) are precursors of dendritic cells. Dendritic cells are antigen-presenting cells and induce T-cell response in both naïve and memory T-cells. The dendritic cells response in melanoma is to induce anti-tumor immunity, which activate neutrophil infiltration to primary melanoma tumors. Neutrophils can switch from tumor suppressive to tumor-promoting type depending on the extracellular stimuli. Growth factor TGF-β has been shown to induce the tumor-promoting phenotype of neutrophils in mesothelioma, but their effect as tumor-promoters is still under investigation (Kitamura, Qian & Pollard, 2015, Botti et al., 2013, Sica et al., 2006).

Stromal myeloid cells have three different subtypes; immature myeloid cells (Gr-1 + CD11b+), tumor-associated macrophages and tumor-associated neutrophils (Yang, Lin, 2017). Tumor-associated macrophages (TAM) are derived from monocyte precursors. Activated TAMs are traditionally divided into two subtypes as proinflammatory M1- and immunosuppressive M2-type macrophages. Tumor cells attract monocytes at the tumor site by secreting chemokines such as CCL-2, CCL-7 and CCL-8, but also VEGF and M-CSF secreted by tumor cells increase macrophage recruitment. In melanoma, especially IL-8, CXCL-12, CCL-2 and CCL-5 types of chemokines promote the inflammatory state of the TME (Sica et al., 2006). Macrophages at the tumor microenvironment can be polarized to tumor-suppressive (M1-type) or to tumor-promoting state (M2-type) depending on the factors secreted by the tumor or stromal cells (Kitamura, Qian & Pollard, 2015). M1-type TAMs produce proinflammatory cytokines such as IL-1β, IL-6, TNF-α and are cytotoxic for neoplastic cells, whereas M2-type TAMs are tumor promoters with immunosuppressive effects and secrete anti-inflammatory cytokines such as IL-4, IL-10, M-CSF, chemokines such as CXCL-2 and CXCL-8. In addition, these immunosuppressive M2-type TAMs promote angiogenesis by secreting VEGF-A (Sica et al., 2006). Polarized M2-type TAMs are able to suppress cytotoxic T-cells (CD8+) activity by ligand binding, such as via PD-L1. Receptor for PD-L1, PD-1, is expressed at the cell surface of activated T-cells, as well as in NK cells and B-cells. PD-1 expression in T-cells and its ligation to PD-L1 on tumor cell surface enables tumor cells escape from immune recognition (Simon, Labarriere, 2017). Also macrophages express PD-L1 on their cell surface, and activated M2-type macrophages are able to hinder T-cell function and further immunosuppression in melanoma (Cao et al., 2017). Furthermore, the tumor-promoting state is further maintained by proinflammatory factors such as, VEGF-A, IL-1, IL-6, IL-17 and CXCL-1 secreted by tumor and stromal cells and macrophages (Blank et al., 2016).

**Tumor stroma - cancer-associated fibroblasts**

Stromal cells surrounding the tumor consist mainly of fibroblasts. These active stromal fibroblasts are called myofibroblasts, or in cancer stroma, so-called CAFs. The population of CAFs is heterogeneous and in addition to stromal fibroblasts, they can originate from adipocytes, bone marrow derived hematopoietic and mesenchymal stem cells, epithelial cells and endothelial cells (Shiga et al., 2015). The CAFs are important promoters of cancer progression and modifiers of the ECM, which stimulate tumor cell proliferation, migration and invasion, and even induce EMT changes in cancer cells (Augsten, 2014). Cytokines such as IL-6, VEGF and C-MSF produced by CAFs can modulate the TME immunosuppressively by activating MDSC differentiation, thereby favoring cancer progression (Mace et al., 2013). The
CAFs start to express α smooth muscle actin (α-SMA) and fibroblast activation protein (FAP), which are indicators of transformed fibroblasts (Kalluri, Zeisberg, 2006). Other specific CAF markers are fibroblast-specific protein 1 (FSP-1/S100A4), neuron glial antigen-2 (NG2), tenascin C, desmin, vimentin, the expression of growth factor receptors PDGFRα and –β (Augsten, 2014), secretion of cytokines, chemokines, growth factors and MMP-2 and MMP-9; these factors degrade basement membrane collagen type IV and laminin which enhances tumor cell invasion and metastasis (Shiga et al., 2015, Augsten, 2014).

Before the transformation to CAFs, normal fibroblasts can restrict the development of cancer. Recently Zhou et al (2016) showed that dermal fibroblasts induce cell cycle arrest in early stage melanoma cells and inhibit EMT. Normal fibroblasts increase melanoma cell p16 expression and decrease cyclin D1 expression, which together reduce melanoma cell proliferation (Zhou et al., 2016). On the other hand, after the transformation of dermal fibroblasts to CAFs, they can be the driving force of melanoma metastasis. This accentuates the importance of growth factor crosstalk between cancer cells and stromal cells. Culture media collected from the melanoma cells containing PDGF-AA and PDGF-CC, activated the expression of HAS2 and hyaluronan production in dermal fibroblasts in a PDGFR-PI3K-AKT manner thereby enhancing melanoma cell proliferation and MMP-2 and MMP-9 expression (Willenberg et al., 2012). Another growth factor important in the crosstalk between melanoma cells and fibroblasts is TGF-β. TGF-β can act as an inhibitory as well as activating factor in melanoma progression, depending on the cells differentiation stage (Bierie, Moses, 2006). Izar et al. (2016) showed that melanoma cells exclusively produce TGF-β, which induces the formation of CAFs, and as a result, promotes melanoma cell proliferation, invasion (Izar et al., 2016, Yin et al., 2012) and survival (Berking et al., 2001). TGF-β induced CAFs have been shown to produce an immunosuppressive microenvironment by inducing polarization of TAMs towards a protumoral phenotype (M2) (Takahashi et al., 2017, Berking et al., 2001). Different cell types, secreted factors and the ECM components involved in the TME are represented in figure 5.

![Figure 5. Schematic presentation of the cells and their secreted growth factors in the tumor microenvironment (modified from Hsu et al. 2002).](image)

The crosstalk between melanoma cells and CAFs can also be mediated by exosomes and microvesicles secreted by the melanoma cells. Melanoma cell-derived exosomes contain specific
markers such as melanoma-associated antigens, NRAS and Src that can transfer oncogenic signals to the surrounding cells and favor tumor escape (Lazar et al., 2015). Microvesicles released by the melanoma cells transformed normal fibroblasts to CAFs via activation of the ERK1/2 pathway and VCAM-1 expression (Zhao et al., 2015). Melanoma cells can secrete proteolytically active MMP-14 in exosomes to modulate the content of ECM (Hakulinen et al., 2008); this secretion can also act vice versa. Microvesicles secreted by CAFs transfer proteins and lipids in to human prostate cancer and melanoma cells which support their growth (Santi et al., 2015). Also melanosomes released by the melanoma cells can carry MiR-211, which can activate dermal fibroblasts via the IGF2R-MAPK signaling pathway to produce IL-1β, IL-6, IL-8, CXCL-1, CXCL-2 and COX-2, modulating the ECM to favor for melanoma invasion (Dror et al., 2016).

2.4.2 Epithelial-mesenchymal like transition in melanocytes

Epithelial-mesenchymal transition, EMT, is a normal process during morphogenesis and wound repair, but also occurs in pathological situations such as fibrosis and cancer (De Craene, Berx, 2013). In cancer progression, the loss of E-cadherin, a cell-cell adhesion molecule, and the loss of apical-basal polarity and the increased cellular motility are involved in the EMT. E-cadherin (CDH1) regulates the epithelial homeostasis, controls the expression of desmosomal proteins, tight junction proteins and cell polarity proteins. Transition to a mesenchymal phenotype involves gaining the expression of N-cadherin, vimentin, fibronectin and MMPs (Lee, Herlyn, 2007). TGF-β induces EMT in numerous cell lines, such as in bovine mammary gland epithelial cells in vitro via the TGF-β1/SMAD signaling pathway (Chen et al., 2017).

For full EMT, it is not enough that only one or two changes occur; instead collective changes in transcription factors such as ZEB2, TWIST1 expression by activated RAS trigger cellular reprogramming (Morel, 2012). Melanocytic cells which are not epithelial cells, do not undergo the normal type of EMT, neither are all the EMT-like changes yet uncovered in melanoma progression. The first EMT-type change that occurs in melanoma is the loss of E-cadherin expression coupled with the increased β-catenin signaling (Lee, Herlyn, 2007) and the expression of EMT transcription factors SNAI1 (Poser et al., 2001), SLUG, ZEB1 and ZEB2 (De Craene, Berx, 2013). These transcription factors can repress several adhesion and junction proteins, such as claudins and desmosomal proteins (Vandewalle et al., 2005). SNAI1 binds to the E-cadherin promoter site CDH1 and represses its transcription (Batlle et al., 2000). N-cadherin, SPARC and WNT receptor Frizzled were identified as changed EMT markers in a high-throughput study of primary VGP melanoma patients (Alonso et al., 2007). This study indicated that N-cadherin expression is associated with the loss of type II cadherin 10 (CDH10).

Epithelial-mesenchymal transition changes have been connected to hyaluronan metabolism, as shown with HAS1 (Nguyen et al., 2017) and HAS2 (Preca et al., 2017) and with excessive production of hyaluronan (Koyama et al., 2007). In all of these cases, hyaluronan or HAS overexpression induces loss of epithelial traits and cell-cell adhesion. Normal heart morphogenesis in mice shows the essential function of Has2 in cardiac cushion tissue and in the transformation of endocardial cells into mesenchymal cells (Camenisch et al., 2000). In zebrafish epicardial heart regeneration following injury, the increased expression of hyaluronan, hyaluronan synthases 1 and 2 and hyaluronan receptor RHAMM were essential for proper renewal. Inhibition of hyaluronan production following injury reduced epicardial cell EMT, cell migration to the injury area and coronary vasculature formation (Missinato et al., 2015). In epicardial cell EMT during differentiation, TGF-β induces signaling via MEKK3-ERK1/2, which leads to Has2 expression (Craig et al., 2010). In rat primary mesothelioma cells, EGF or wound healing induced EMT is associated with increased CD44 expression and hyaluronan synthesis (Koistinen et al., 2017).

In breast cancer cells, tumor-derived hyaluronan activated ZEB1 transcription, which in turn activated hyaluronan synthesis via upregulation of HAS2; this result revealed, for the first time, the autocrine mechanism for hyaluronan-ZEB1-HAS2 that accelerates EMT changes in breast
cancer cells (Preca et al., 2017). HAS2 has been shown to induce EMT in NMuMG mammary epithelial cells via TGF-β-SMAD-p38 signaling and suppression of HAS2 could reduce the EMT transcription factors SNAI1 and ZEB1 (Porsch et al., 2013). Hyaluronan receptor CD44 has been associated with EMT changes in colon cancer invasion via CD44-dependent downregulation of E-cadherin and upregulation of N-cadherin, vimentin, fibronectin and MT1-MMP (Cho et al., 2012). HAS3 overexpression in an epithelial lung adenocarcinoma cell line resulted in an EMT cell phenotype, with enhanced MMP-2 and MMP-9 expression and cell invasive capacity. Similar effects were obtained with exogenous hyaluronan (Chow, Tauler & Mulshine, 2010). In contrast, hyaluronan has been shown to have a negative impact on EMT as well, since hyaluronan oligosaccharides can induce VEGF secretion and attenuate cardiac development (Rodgers et al., 2006). Moreover, hyaluronan and HASes may possess other, yet undiscovered effects on EMT.

2.4.3 Tumor-stroma interaction in cancer development and progression

Stephen Paget postulated in his “seed and soil” hypothesis already in 1889 that cancer cells can only colonize to tissues that are permissive to growth (Fidler, 2003). More recent studies about the tumor-stroma interaction support this old theory indicating that the driving force for cancer metastasis comes from the stromal cells rather than from the tumor cells (Bissell, Hines, 2011, Lee, Herlyn, 2007, Kalluri, Zeisberg, 2006). In cutaneous melanoma, the tumor and the stroma are a heterogeneous population of cells; tumor cells in the center and activated fibroblasts in the invasive front are surrounded by a poorly defined peritumoral zone. The tumor stroma in cutaneous melanoma can be desmoplastic which is defined by fibroblasts and fibrocytes accompanied with fibrillar ECM components. Alternatively, it can be myxoid, where atypical spindle cells fill the ECM rich in proteoglycans (Ruiter et al., 2002). The first steps in the tumor-stroma interaction are the secretion of growth factors such as bFGF, PDGF and cytokine TGF-β from melanoma cells that activate fibroblasts. The stromal fibroblasts are activated to proliferate and produce ECM components such as collagen, fibronectin, hyaluronan, laminin and osteopontin (Villanueva, Herlyn, 2008, Ruiter et al., 2002). Secretion of VEGF by the melanoma cells induces surrounding endothelial cell neovascularization; this provides a way for immune cells to infiltrate to the tumor vicinity (Ruiter et al., 2002). Growth factors induce signaling pathways such as focal adhesion kinase (FAK), which in cancer cells, increases their migration via RAS-mediated MAPK-ERK activation. Activation of the PI3K-AKT pathway suppresses apoptosis and increases tumor cell survival (Liotta, Kohn, 2001).

As discussed in section 2.4.1 Tumor ECM and in Tumor stroma - immune cells, immune cells in the tumor vicinity accelerate the acquisition of the immunosuppressive, tumor promoting state. IL-4 and IL-13 secreted by the tumor cells induce monocytes to transform to M2-type polarized macrophages, whereas secreted IL-10 from tumor cells further induces M2-type macrophages to remodel the ECM and favor tumor invasion (Sica et al., 2006). Not only the tumor cells, but also the CAFs can induce M2-type macrophages and promote the immunosuppressive TME. Recently Takahashi et al. (2017) reported that CAFs from oral squamous cell carcinoma suppressed proliferation of T-cells by promoting M2-type macrophage polarization. High infiltration of CAFs correlated with M2-type macrophages together with lymph node invasion and TNM stage (Takahashi et al., 2017). ECM remodeling involves secretion of active MMPs by CAFs, which can decrease the susceptibility of melanoma cells to NK cell-mediated cytolysis. MMPs secreted by CAFs induce the shedding of MICA/B from the melanoma cells surface, which prevents the recognition of melanoma cells by NK-cells (Ziani et al., 2017).

Cancer cells need to induce angiogenesis and activate the vasculature to supply oxygen and nutrients to the tumor site. A new vasculature network, neovasculature, is formed from the pre-existing ones via activated stromal endothelial cells. Throughout this neovasculature cancer cells can also metastasize to distant locations (Wang et al., 2017a). Tumor vasculature is disorganized and leaky due to altered endothelial cell adherens junctions and tight junctions, which are critical to maintain the vascular barrier functions (Yang, Lin, 2017). Tumor cells
secrete pro-angiogenic factors such as VEGF, bFGF, PDGF and placental growth factor (PIGF), activate endothelial cells to migrate, proliferate and start neovasculogenesis. These growth factors signal through endothelial cell receptors such as VEGF receptors (VEGFR) 1 and 2 (Wang et al., 2017a, Bergers, Benjamin, 2003). VEGF activates endothelial FAK, which promotes the dissociation of VE-cadherin from β-catenin, the breakdown of adherens junctions between endothelial cells and promotes angiogenic switch (Yang, Lin, 2017).

The balance between pro- and anti-angiogenic signals determines the impact of new neovasculature. p53 regulates several anti-angiogenic proteins such as thrombospondin-1 (TSP-1), brain-specific angiogenesis inhibitor 1 (BAII) and MMP-2, which promote anti-angiogenic peptide formation from basement-membrane proteins at the early stages of tumor neovascularization (Bergers, Benjamin, 2003). Thrombospondin-1 paracrinically signals in tumor cells via bone morphogenetic protein 4 (BMP4) and downregulates VEGF expression from tumor cells, thereby suppressing angiogenesis (Tsuchida et al., 2014).

UVB, TGF-β and hypoxia induce melanoma cells to secrete IL-8, which in turn activate endothelial cells migration and modulate vascular permeability. Increased anti-apoptotic Bcl-xL and IL-8 expression from melanoma cells also enhance tumor cells dissemination through the stromal vasculature network (Gabellini et al., 2018, Villanueva, Herlyn, 2008). In endothelial cells, VEGF and bFGF increase the binding of integrin αvβ3 to vitronectin or osteopontin, which promote angiogenesis. Melanoma cells secrete MMPs such as -1, -2, -9, -13, and -14 and TIMP-1, -2 and -3 that promote angiogenesis in tumor stroma by modulating the ECM permeable for VEGF allowing it to bind to its receptor (Villanueva, Herlyn, 2008, Bergers, Benjamin, 2003). Tumor cells promote endothelial cell necrosis via death receptor 6 causing increased leakiness in the vasculature, which allows the tumor cells to extravasate and metastasize (Strilic et al., 2016).

### 2.5 HYALURONAN IN CANCER

#### 2.5.1 Involvement of hyaluronan in cancer

The involvement of hyaluronan in different cancers has been studied intensely for decades. Depending on the tissue origin of the tumor or stromal impact on hyaluronan synthesis, its synthases or hyaluronidases are involved in many types of cancers. Either the increase or the decrease of hyaluronan in the tumor tissue or stromal tissue correlates with the poor prognosis in several cancers, and can be used as a prognostic marker for patient survival and cancer recurrence (Sironen et al., 2011, Tammi et al., 2008). Different types of cancers are listed in table 1 with the expression (high/low) of hyaluronan and related enzymes and the clinical outcome.

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>Cell type</th>
<th>HA/HASes, HYAL</th>
<th>Expression</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-cell non-Hodgkin</td>
<td>S</td>
<td>HA/HYAL2</td>
<td>↑/↓</td>
<td>poor prognosis</td>
<td>Bertrand et al., 2005</td>
</tr>
<tr>
<td>lymphoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bladder cancer</td>
<td>T</td>
<td>HAS1, HAS2, HYAL1</td>
<td>↑/↑/↑</td>
<td>predictor of metastasis, recurrence</td>
<td>Kramer et al., 2011</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>HYAL1</td>
<td>↑</td>
<td>muscle invasion, recurrence</td>
<td>Kramer et al., 2010</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>HAS1</td>
<td>↑</td>
<td>recurrence</td>
<td>Golshani et al., 2007</td>
</tr>
</tbody>
</table>

Table 1. Hyaluronan, HAS and HYAL expression in different malignancies. Used markings describes the tissue type; tumor tissue (T), stromal tissue (S) and expression high (↑) or low (↓).
<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Tumor Site</th>
<th>HA Protein</th>
<th>Variation</th>
<th>Prognosis</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast cancer</td>
<td>T, S</td>
<td>HA</td>
<td>↑/↑</td>
<td>Poor prognosis</td>
<td>Auvinen et al., 2000</td>
</tr>
<tr>
<td>AR’ breast cancer</td>
<td>T</td>
<td>HAS2</td>
<td>↑</td>
<td>Poor prognosis</td>
<td>Zhang et al., 2016</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>HAS2</td>
<td>↑</td>
<td>Tumorigenesis, metastasis</td>
<td>Li et al., 2015</td>
</tr>
<tr>
<td></td>
<td>T+S</td>
<td>HAS1-3</td>
<td>↑</td>
<td>Poor prognosis</td>
<td>Auvinen et al., 2014</td>
</tr>
<tr>
<td>Cutaneous melanoma</td>
<td>T</td>
<td>HAS1/HAS2</td>
<td>↓/↓</td>
<td>Poor prognosis</td>
<td>Poukka et al., 2016</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>HA</td>
<td>↓</td>
<td>Poor prognosis</td>
<td>Karjalainen et al., 2000</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>T</td>
<td>HA</td>
<td>↑</td>
<td>Poor prognosis</td>
<td>Ropponen et al., 1998</td>
</tr>
<tr>
<td>Endometrial cancer</td>
<td>T</td>
<td>HYAL1</td>
<td>↓</td>
<td>Early recurrence</td>
<td>Nykopp et al., 2015</td>
</tr>
<tr>
<td>Epithelial ovarian cancer</td>
<td>S</td>
<td>HA</td>
<td>↑</td>
<td>Poor prognosis</td>
<td>Anttila et al., 2000</td>
</tr>
<tr>
<td>Lung adenocarcinoma</td>
<td>S</td>
<td>HA</td>
<td>↑</td>
<td>Poor prognosis</td>
<td>Pirinen et al., 2001</td>
</tr>
<tr>
<td>Oral squamous cell carcinoma</td>
<td>T</td>
<td>HA</td>
<td>↓</td>
<td>Poor prognosis</td>
<td>Kosunen et al., 2004</td>
</tr>
<tr>
<td>Pancreatic ductal adenocarcinoma</td>
<td>T</td>
<td>HA</td>
<td>↑</td>
<td>Poor prognosis</td>
<td>Cheng et al., 2013</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>T</td>
<td>HA</td>
<td>↑</td>
<td>Poor prognosis</td>
<td>Posey et al., 2003</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>HA</td>
<td>↑</td>
<td>Recurrence</td>
<td>Aaltomaa et al., 2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Poor differentiation, metastases</td>
<td>Lipponen et al., 2001, Lokeshwar et al., 2001</td>
</tr>
<tr>
<td>Thyroid carcinoma</td>
<td>S</td>
<td>HA</td>
<td>↑</td>
<td>Poor prognosis</td>
<td>Böhm et al., 2002</td>
</tr>
<tr>
<td>Urothelial carcinoma</td>
<td>T</td>
<td>HAS3</td>
<td>↓</td>
<td>Poor prognosis</td>
<td>Chang et al., 2015</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>plasma</td>
<td>HA</td>
<td>↑</td>
<td>Marker for metastasis</td>
<td>Peng et al., 2016</td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>serum</td>
<td>HA</td>
<td>↑</td>
<td>Poor prognosis</td>
<td>Mima et al., 2014</td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>serum</td>
<td>HA</td>
<td>↓</td>
<td>Shorter survival</td>
<td>Dahl et al., 1999</td>
</tr>
</tbody>
</table>

Tumors originating from simple types of epithelium are associated with increased tumor cell-associated hyaluronan. Cell-associated hyaluronan in these cancers also correlates with the tumor invasion, lymph node metastases, angiogenesis as well as local and distant metastases.
The accumulation of hyaluronan in the tumor stroma also correlates with tumor cell invasion in breast, ovarian and prostate cancers (Lipponen et al., 2001, Auvinen et al., 2000, Anttila et al., 2000). Stromal hyaluronan is often an indicator of poor patient outcome, especially in breast cancer (Auvinen et al., 2000). Growth factor signaling, such as EGF, KGF and PDGF, between cancer cells and stromal cells, such as fibroblasts, is the major factor producing the hyaluronan-rich matrix (Jacobson et al., 2000, Karvinen et al., 2003b). This provides a favorable niche for tumor growth (Tammi et al., 2008).

The tumor promoting impact of hyaluronan is mediated in different ways. In proliferating tumor cells, hyaluronan acts as a mitogen and inhibition of HAS2 in breast cancer cells in vitro arrests cells to the G0-G1 phase (Udabage et al., 2005). Hyaluronan facilitates cellular adhesion but also detachment during mitosis (Brecht et al., 1986). Inhibition of HAS2 induces fibroblast senescence in pulmonary fibrosis via the p27-CDK2-SKP2 pathway (Li et al., 2016) and cell cycle arrest with apoptosis in breast cancer (Li et al., 2015). In mice, increased Has2 expression and hyaluronan synthesis induces EMT by downregulation of E-cadherin and enhanced nuclear translocation of β-catenin (Itano et al., 2002). Hyaluronan provides a hydrated extracellular milieu for enhanced cell migration, invasion and growth via activation of its receptors CD44 or RHAMM (Brett et al., 2018, Shigeeda et al., 2017, Zhang et al., 2016, Knudson, 1996). It also contributes to cell proliferation and metastasis by reducing the contact inhibition (Itano et al., 2002). Hyaluronan also promotes drug resistance. Like in multiple myeloma, hyaluronan and proteoglycan link protein 1 (HAPLN1) induced bortezomib-drug resistance via activation of the NF-κB pathway (Huynh et al., 2018), whereas in head and neck cancer stem cells hyaluronan-CD44 mediated signaling induced chemoresistance (Bourguignon, Earle & Shiina, 2017).

Interestingly, tumors arising from stratified epithelia show a different trend compared to cancers originating from simple epithelia. Stratified epithelial cells normally produce high amounts of hyaluronan and are surrounded by a hyaluronan-rich pericellular coat, which even increases in the dysplastic state (Siiskonen et al., 2013, Kosunen et al., 2004). Similar to melanoma (Karjalainen et al., 2000) or SCC (Karvinen et al., 2003a), histochemical staining of hyaluronan in the tumor state in these epithelia showed a decreasing trend during tumor progression. It also correlated with the poor prognosis in oral squamous cell carcinoma (Kosunen et al., 2004) and with increased recurrence in melanoma (Poukka et al., 2016). Low molecular weight hyaluronan in melanoma mice models was shown to induce melanoma lymph node metastasis (Du et al., 2016); this is interesting since another study in mice showed that hyaluronan oligosaccharides (HA12) reduced subcutaneous melanoma tumor formation by inferring with the hyaluronan-CD44 interaction (Zeng et al., 1998). Even in cancers with high tumor cell-associated hyaluronan, the LMW hyaluronan can promote the invasive potential of tumor cells. This was recently reported in 2D and 3D hyaluronan-rich microenvironment studies in melanoma cells, as well as in breast cancer cells. In melanoma, hyaluronan of 30–50 kDa induced melanoma cell proliferation in a CD44-dependent manner (Sapudom et al., 2017). In breast cancer model, hyaluronan of 35 kDa induced cell invasion, MMP9 expression and decreased E-cadherin expression, whereas hyaluronan of 117 kDa produced the opposite effect (Zhao et al., 2017). Low molecular weight hyaluronan (<50 kDa) in breast cancer patients serum was also proposed to be used as a prognostic marker for metastasizing breast cancer (Wu et al., 2014).

Pericellular hyaluronan around cancer or stromal cells, bound to CD44, TSG-6, versican, LYVE-1, RHAMM, or to signal via TLR-4 or -6, can modify the surrounding microenvironment, induce intracellular signaling, stimulate growth and migration of stromal cells or the cancer cells (Chanmee, Ontong & Itano, 2016, Itano, Zhuo & Kimata, 2008). Depending on the cell type, hyaluronan itself or the upregulation or downregulation of HASes can promote cancer progression. Hyaluronan or HASes can be used as a prognostic marker for patient prognosis depending on the cancer (Poukka et al., 2016, Auvinen et al., 2014, Kramer et al., 2011). Secreted factors from tumor cells induce HAS2 expression in stromal cells resulting in elevated hyaluronan production (Willenberg et al., 2012). In inflammation and under stress, cells form...
special cable-like structures that seem to be a way by which inflammatory cells are bound to the tissue (de la Motte et al., 2003). In addition to hyaluronan they can contain TSG-6, Ia1 and versican. Such cables were also formed in keratinocytes by tunicamycin or IL-1β induces stress, leading to enhanced binding of monocytes (Jokela et al., 2008b). Although first described *in vitro*, similar structures were also found in inflamed tissues, such as in colon (Kessler et al., 2008). Also, stromal hyaluronan produced by HAS2 was shown to activate tumor-associated macrophages (TAMs) and regulate the neovascularization in TME (Kobayashi et al., 2010). In breast cancer, the macrophage number, particularly the M2 phenotype, correlates with high tumor hyaluronan content and poor prognosis (Zhang et al, 2017; Tiainen et al., 2014). Recently, it was shown that breast cancer -derived hyaluronan can induce polarization of macrophages towards the M2 phenotype (Zhang et al, 2017).

The molecular size of hyaluronan influences cellular responses, for example HMW hyaluronan has been shown to prevent cancer development in naked mole rats (Tian et al., 2013). On the other hand, LMW hyaluronan induces angiogenesis (Cui et al., 2009), TLR-mediated signaling and promotes cell survival (Yang et al., 2010a), IL-6 and chemokine secretion from dermal fibroblast (Vistejnova et al., 2014) and MMP-9 and MMP-13 production (Fieber et al., 2004). In breast cancer, LMW hyaluronan correlated with immunosuppressive tumor stroma and increased cancer progression. *In vitro* studies indicated that the presence of LMW hyaluronan was due to elevated HYAL-1 and -2 expression. Increased secretion of HYAL-1 and -2 was also detected in the serum of breast cancer patients (Wu et al., 2015).

2.5.2 Hyaluronan in melanoma

The role for hyaluronan in melanoma progression is still unresolved. Melanoma differs from tumors originating from the simple epithelium; in the latter case, cell-associated hyaluronan in cancer is a strong indicator of unfavorable clinical outcome (Tammi et al., 2008). Melanocytes have been shown to produce higher amounts of hyaluronan *in vitro* and other glycosaminoglycans compared to melanoma cell lines (Deen et al., 2016, Bhavanandan, 1981). Hyaluronan metabolism in melanoma progression is similar to cancers originating from the stratified epithelium, such as laryngeal and oral carcinoma. In both of these cancers, reduced hyaluronan in tumor tissue correlates with metastasis and poor patient outcome (Tammi et al., 2008). Poukka (2016) and Siiskonen (2013) have shown that during melanoma progression, the hyaluronan content in the melanoma cells decreases (Siiskonen et al., 2013) and the loss of HAS1 and 2 is associated with increased recurrence (Poukka et al., 2016). Benign nevi embody hyaluronan and its expression is further increased in dysplastic nevi and melanoma *in situ*. In invasive melanoma, hyaluronan content declines; the tumor tissue is almost negative for hyaluronan, which may be due to increased expression of HYAL-2 (Poukka et al., 2016, Siiskonen et al., 2013). The reduction of CD44 and hyaluronan is also shown in clinical stage I cutaneous melanoma and is associated with poor prognosis (Karjalainen et al., 2000).

The effect of hyaluronan and hyaluronan synthases has also been studied *in vitro* in cell cultures. Interestingly, these studies often show opposing results compared to melanoma patient studies. Katona (2016) reported that cultured primary melanocytes only secrete low amounts of hyaluronan and the expression levels of HAS2 and HAS3 increase differently between metastatic melanoma cell lines WM35 and HT168. HAS2 showed stronger protein expression in the WM35 cell line, whereas HAS3 was profoundly expressed in the HT168 cell line. Expression of HAS2 and HAS3 in these cell lines was affected by inhibition of ERK1/2 signaling, which increased hyaluronan synthesis due to elevated HAS3 expression (Katona et al., 2016). Deen (2016) on the other hand showed that hyaluronan secretion was highest in cultured primary melanocytes and decreased towards metastatic melanoma (Deen et al., 2016). Transfection of HAS1 and HAS2 to the metastatic melanoma cell line induced the formation of the pericellular hyaluronan coat and increased cell migration (Ichikawa et al., 1999). Studies in 3D-cultures containing either immobilized or soluble LMW (30–50 kDa) or HWM (500–750 kDa) hyaluronan, showed that BRO melanoma cells proliferated faster in the presence of soluble
LMW hyaluronan, whereas invasion was enhanced by the immobilized LMW hyaluronan; in both cases, the stimulation was dependent on CD44. High molecular weight hyaluronan had no influence (Sapudom et al., 2017). The study from Kim and coworkers (2007) supports the theory that external hyaluronan increases melanoma cell migration. Exogenously added hyaluronan (1 MDa) increased the invasion and migration of B16-F10 melanoma cells through CD44 signaling and induced upregulation of osteopontin and transglutaminase II via NF-κB activation (Kim et al., 2008). These contradictory results may be the sum of different factors; perhaps the specific cell lines that were used, as well as impurities in the exogenously added LMW and HMW hyaluronan, such as LPS and growth factors.
3 Aims of the study

Hyaluronan, a long linear extracellular matrix molecule, is associated with many cellular functions such as proliferation, migration, invasion, angiogenesis, signaling etc. It is synthesized by three hyaluronan synthases, each having their own unique function. The equilibrium between hyaluronan synthesis and degradation maintains the delicate balance; disturbances in this balance can lead to inflammation and eventually cancer progression. The role of hyaluronan in human malignancies is widely studied and its upsurge in either tumor cells or stromal cells predicts poor prognosis in many type of cancers. In melanoma, the influence of hyaluronan in melanomagenesis is not so well-known. Benign nevi and primary tumors (melanoma in situ) express high levels of hyaluronan, compared to deep metastatic melanomas. The main aims of this thesis were to study how hyaluronan affects melanoma progression from primary melanocytes to metastatic melanoma and to explore how the changes in hyaluronan metabolism initiate melanomagenesis. In addition to these aims, the thesis will address how the secreted factors from melanoma cells modify surrounding fibroblasts in cancer stroma.

The specific aims of the theses were:

1. To study the influence of the hyaluronan coat in primary melanocytes in the protection against UVB radiation and how the degradation of this coat affects melanocyte viability and transformation to melanocytic cells after UVB.

2. To study the influence of HAS3 overexpression in metastatic melanoma cell line, and how increased hyaluronan synthesis affects melanoma cell proliferation, migration and adhesion ability.

3. To investigate the effect of growth factors and cytokines secreted by the melanoma cells on fibroblasts and to address the question; are melanoma cells able to activate dermal fibroblasts to cancer-associated fibroblast via soluble factors?
4 Materials and methods

4.1 MATERIALS

4.1.1 Cell lines
Table 2 shows the cell lines used in this thesis and the specific culture conditions are presented in the original publications.

Table 2. Cell lines used in the publications.

<table>
<thead>
<tr>
<th>Name of the cell line</th>
<th>Abbreviation</th>
<th>Original publication</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human dermal fibroblasts</td>
<td>Fibroblast</td>
<td>III</td>
<td>Dr. Michael Edward, Univ. of Glasgow, UK</td>
</tr>
<tr>
<td>Human epidermal melanocytes, adult</td>
<td>HEMa</td>
<td>I</td>
<td>ScienCell, #2230</td>
</tr>
<tr>
<td>Human metastatic melanoma cell line</td>
<td>C8161</td>
<td>II, III</td>
<td>Welch et al., 1991</td>
</tr>
<tr>
<td>Human metastatic melanoma cell line</td>
<td>MV3</td>
<td>II</td>
<td>van Muijen et al., 1991</td>
</tr>
<tr>
<td>Human metastatic melanoma cell line with doxycycline-inducible overexpression of EGFP-HAS3</td>
<td>EGFP-HAS3-C8161</td>
<td>II</td>
<td>Dr. Genevieve Bart, Univ. of Eastern Finland</td>
</tr>
<tr>
<td>Human metastatic melanoma cell line with doxycycline-inducible overexpression of EGFP-HAS3</td>
<td>EGFP-HAS3-MV3</td>
<td>II</td>
<td>Dr. Genevieve Bart, Univ. of Eastern Finland</td>
</tr>
</tbody>
</table>

4.1.2 Specific reagents used
The pericellular hyaluronan coat was degraded with Streptomyces hyaluronidase (H1136, Sigma-Aldrich, St. Louis, MO) 1 TRU/ml in the original publications (I-II). Hyaluronan secretion was measured using an in-house sandwich type hyaluronan-ELSA (enzyme-linked sorbent assay) and visualized with either an in-house made biotinylated HABR (bHABC) for fixed cells or an Alexa fluor 568-labelled HABR (fHABC) for live cell imaging with confocal microscopy (Rilla et al., 2008, Hiltunen et al., 2002, Tammi et al., 1994). An angiogenesis array (ARY007), cytokine array (ARY005B), phosphokinase array (ARY003B) and phospho-receptor tyrosine kinase array (ARY001B) from R&D Systems were used according to the manufactures protocol in the original publications (I-III). Protein inhibitors were used to block different signaling molecules; PI3K inhibitor Wortmannin (2 µM), AKT kinase inhibitor VIII (1–5 µM), MEK kinase inhibitor UO126 (5 µM), PDGFR inhibitor AG1296 (5 µM), p38 inhibitors SB203580 (15 µM) and BIRB796 (10 µM), NF-κB inhibitor JSH-23 (5 µM), TLR-4 inhibitor TAK-242 (10 µM). All chemicals were purchased from Calbiochem (La Jolla, CA), Sigma-Aldrich (JSH-23 inhibitor) or MedChem Express (Monmouth Junction, NJ, TAK-242 inhibitor). The inhibitors were dissolved in sterile DMSO or water and stored frozen.

Table 3. Primer sequences used in the original publications

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Length</th>
<th>Original publication</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARPO</td>
<td>For 5’ AGATGCAGCAGATCCGCGAT 3’</td>
<td>319 bp</td>
<td>I, II, III</td>
<td></td>
</tr>
<tr>
<td>(RPLP0)</td>
<td>Rev 5’ GTGGTGATACCTAAGCCTG 3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Forward Primer</td>
<td>Reverse Primer</td>
<td>Size (bp)</td>
<td>Tissues</td>
</tr>
<tr>
<td>---</td>
<td>----------------------------------------------------</td>
<td>-----------------------------------------------------</td>
<td>-----------</td>
<td>------------</td>
</tr>
<tr>
<td>HAS1</td>
<td>5' CAAGATTCTTCAGTCTGGAC 3'</td>
<td>5' TAAGAAGCAGAGAAAGCAG 3'</td>
<td>124</td>
<td>I, II, III</td>
</tr>
<tr>
<td>HAS2</td>
<td>5' CAGAATCCAAACAGACAGTTC 3'</td>
<td>5' TAAGGTGTTGTTGTTGACTG 3'</td>
<td>187</td>
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<tr>
<td>HAS3</td>
<td>5' CTTAAGGTTGTTCCTTCGTC 3'</td>
<td>5' GTTCTGGGGAGATGAAAGAA 3'</td>
<td>194</td>
<td>I, III</td>
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<tr>
<td>chHAS3</td>
<td>5' GTCAGTGGTCACGGGTTTCT 3'</td>
<td>5' AGGCAATGAAGTTCACCAC 3'</td>
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<td>II</td>
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<td>5' CACCTACCCCCAGCAACCCTA 3'</td>
<td>5' CTGCTCTGCTCTGCTCGGTGAT 3'</td>
<td>153</td>
<td>I, II, III</td>
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<td>HYAL-2</td>
<td>5' CCTCTGGGGCTTCTACTCTCT 3'</td>
<td>5' CTGAAACAGGAAGCTCACAA 3'</td>
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<td>I, III</td>
</tr>
<tr>
<td>IL-6</td>
<td>5' TGCATATAACCCACCTGCCA 3'</td>
<td>5' GTGCCCATGCTACATTTGCC 3'</td>
<td>163</td>
<td>I</td>
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<tr>
<td>IL-8</td>
<td>5' GAGTGGACCACACTGCAGCA 3'</td>
<td>5' TCCACACCCCTCTGCAACCAATGT 3'</td>
<td>102</td>
<td>I</td>
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<tr>
<td>CXCL-1</td>
<td>5' CCCAAACCGAAGTCATAGCC 3'</td>
<td>5' CAGGAACAGCCACCAGTGA 3'</td>
<td>154</td>
<td>I</td>
</tr>
<tr>
<td>CXCL-10</td>
<td>5' GCCATTCTGATTTGCTGCCAT 3'</td>
<td>5' TGCTCCCCCTCTGCTTCTTAAG 3'</td>
<td>300</td>
<td>I</td>
</tr>
<tr>
<td>MMP-1</td>
<td>5' GAAAGCGGAGAAATAGTG 3'</td>
<td>5' TCCAGGTCCATCAAAAGG 3'</td>
<td>381</td>
<td>III</td>
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<tr>
<td>MMP-2</td>
<td>5' TGCTGGAGACAATATTCTGGA 3'</td>
<td>5' ACCTCACGCTCTTCAGACTTTG 3'</td>
<td>200</td>
<td>III</td>
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<tr>
<td>MMP-9</td>
<td>5' TGCCCGGCCAAGAGTACAG 3'</td>
<td>5' TCAGGCGGAGGACTAGAG 3'</td>
<td>182</td>
<td>III</td>
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<tr>
<td>MMP-14</td>
<td>5' GCCTCTGCTTCTGATAAAC 3'</td>
<td>5' GCATCAATCGTCTGGCTAGG 3'</td>
<td>345</td>
<td>III</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>5' GAAAGCTGCTACCTGCATGA 3'</td>
<td>5' CTTCCTTAGCAGGATCAGC 3'</td>
<td>187</td>
<td>III</td>
</tr>
<tr>
<td>PDGFRβ</td>
<td>5' TGAGACGTGGTCCTCATC 3'</td>
<td>5' GTACTGGGCTCAGCCTCCAG 3'</td>
<td>129</td>
<td>III</td>
</tr>
</tbody>
</table>
4.2 METHODS

4.2.1 UV-exposure
A handheld UV-lamp (UVM-57, 302nm) purchased from Upland, California, USA, was used in melanocyte experiments (I). Irradiance was determined using a Macam SR9910 spectroradiometer (Macam Photometrics Ltd., Livingston, Scotland). Subsequently, the intensity (MED/h) and stability was calibrated using a Solar Light 3D UV-meter (Solar Light Company, Inc., PA, USA). The intensity W/m² was determined using calibration values for the Solar Light 3D meter and the CIE value from Macam SR9910 spectrum for UVM-57 lamp; the intensity was converted to mJ/s/cm². Cells were exposed to UVB under PBS in all experiments.

4.2.2 Biochemical, cellular and molecular biology methods
The general methods used in this thesis are listed in tables 4 and 5. More specific details of every method are presented in the original publications (I-III). Table 4 lists the methods used to study hyaluronan secretion and imaging of pericellular hyaluronan. Table 5 lists general methods used to study cell behavior and gene or protein expression in different original publications.

<table>
<thead>
<tr>
<th>Method</th>
<th>Purpose</th>
<th>Original publication</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Content of hyaluronan (HA-ELSA)</td>
<td>Measure secreted and pericellular hyaluronan</td>
<td>I, II, III</td>
<td>Hiltunen et al., 2002</td>
</tr>
<tr>
<td>Fluorescence immunohistochemistry (fHABC-probe)</td>
<td>Image pericellular hyaluronan in live cells</td>
<td>I, II, III</td>
<td>Rilla et al., 2008</td>
</tr>
<tr>
<td>Histochemistry (bHABC-probe)</td>
<td>Image pericellular and intracellular hyaluronan in fixed cells or tissue sections</td>
<td>I, III</td>
<td>Tammi et al., 1994</td>
</tr>
<tr>
<td>Size exclusion chromatography (gel filtration HPLC)</td>
<td>Determine the molecular mass distribution of native hyaluronan</td>
<td>I, II, III</td>
<td>Tammi et al., 2000</td>
</tr>
</tbody>
</table>
Table 5. Methods used to study cell behavior and gene and protein expression. Specific details are presented 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><strong>General methods</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adhesion</td>
<td>To study cell ability to adhere to collagen matrix after treatment</td>
<td>II</td>
<td>Optimized in original publication II</td>
</tr>
<tr>
<td>Apoptosis (PI staining for FACS)</td>
<td>To study cell cycle and the portion of apoptotic cells</td>
<td>II</td>
<td>Optimized in original publication II</td>
</tr>
<tr>
<td>Invasion</td>
<td>To study cell ability to invade into 3D matrix</td>
<td>III</td>
<td>Optimized in original publication III</td>
</tr>
<tr>
<td>Migration (scratch wound assay, single cell random migration imaging)</td>
<td>To study cell motility after treatment</td>
<td>II</td>
<td>Optimized in original publication II</td>
</tr>
<tr>
<td>Proliferation/cell counting/viability</td>
<td>To study cell proliferation rate after different treatments</td>
<td>II, III</td>
<td>Optimized in original publications II and III</td>
</tr>
<tr>
<td>Scanning electron microscopy, SEM</td>
<td>To study plasmamembrane protrusions and morphological changes in cells</td>
<td>III</td>
<td>Optimized in original publication III</td>
</tr>
<tr>
<td><strong>Gene and protein expression methods</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quantitative real-time PCR (qRT-PCR)</td>
<td>Quantification of mRNA expression</td>
<td>I, II, III</td>
<td>Optimized in original publications I–III</td>
</tr>
<tr>
<td>Western blotting</td>
<td>Quantification of protein expression/protein phosphorylation</td>
<td>I, II, III</td>
<td>Optimized in original publications I–III</td>
</tr>
<tr>
<td>Angiogenesis Array</td>
<td>Quantification of secreted angiogenic factors</td>
<td>III</td>
<td>Optimized in original publication III</td>
</tr>
<tr>
<td>Cytokine Array</td>
<td>Quantification of secreted cytokines</td>
<td>I, III</td>
<td>Optimized in original publications I and III</td>
</tr>
<tr>
<td>Phosphokinase Array</td>
<td>Quantification of activated phosphokinases</td>
<td>II, III</td>
<td>Optimized in original publications II and III</td>
</tr>
<tr>
<td>Phospho-receptor tyrosine kinase Array</td>
<td>Quantification of activated tyrosine kinase receptors</td>
<td>III</td>
<td>Optimized in original publication III</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>To study protein distribution and localization (actin, CD44, HAS2, vinculin)</td>
<td>II, III</td>
<td>Optimized for every cell line in original publications II and III</td>
</tr>
</tbody>
</table>
5 Results

5.1 THE DIFFERENTIAL HYALURONAN SYNTHESIS IN MELANOCYTES, FIBROBLASTS AND MELANOMA CELL LINES

In the present thesis primary melanocytes and fibroblasts, as well as melanoma cell lines C8161 and MV3, were used to study the effect of hyaluronan in the early phase of melanomagenesis, and in melanoma cell behavior and in the tumor-stroma interaction. While primary melanocytes and fibroblasts produced ~35 ng/10,000 cells of hyaluronan, its production was much lower in melanoma cells lines. MV3 melanoma cells secreted ~7 ng/10,000 cells of hyaluronan, whereas C8161 melanoma cells produced ~1 ng/10,000 cells. Live cell imaging using a confocal microscope confirmed that primary melanocytes and fibroblasts contain much larger pericellular coats compared to MV3 or C8161 melanoma cell lines used in this thesis (Fig. 6). In all the cell lines used in these studies, HASes were expressed and HAS2 was the most expressed in melanocytes, fibroblasts and MV3 cells, whereas HAS1 was expressed at the lowest level. However, the expression level of HAS3 was highest of all the HASes in C8161 cells, compared to the other cell lines. All cell lines also expressed CD44 and HYAL-2 (Table 6).

Figure 6. The pericellular hyaluronan coat in the cell utilized in this thesis. The pericellular hyaluronan coat was probed with fHABC-568 (red) and nuclei (blue) either with DraQ5™ or NucBlue™ and live cells were imaged with confocal microscopy. Higher magnification of a single cell in MV3 melanoma cell image (scale bars represent 20 µm).
Table 6. Ct-value ranges of HAS1–3, CD44 and HYAL-2 from qRT-PCR experiments from different cell lines used in the original publications.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>HAS1</th>
<th>HAS2</th>
<th>HAS3</th>
<th>CD44</th>
<th>HYAL-2</th>
<th>Publication</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEMa</td>
<td>27–30</td>
<td>22–24</td>
<td>27–30</td>
<td>18–20</td>
<td>21–24</td>
<td>I</td>
</tr>
<tr>
<td>with doxycycline induction (0.05–0.5 µg/ml)</td>
<td></td>
<td></td>
<td></td>
<td>14–17</td>
<td></td>
<td>II</td>
</tr>
</tbody>
</table>

5.2 HYALURONAN IN UV-RESPONSE IN PRIMARY MELANOCYTES

5.2.1 UVB decreases hyaluronan secretion in melanocytes
As shown from hyaluronan stained benign nevi samples, melanocytes also secrete high levels of hyaluronan in the cell culture conditions. Live cell imaging with fHABC confirmed a large pericellular hyaluronan coat surrounds primary melanocytes (publication I, figure 1A-C). UVB-exposure of different doses (0–70 mJ/cm²) caused a dose-dependent decrease in the amount of hyaluronan secreted to the culture medium; a 30 mJ/cm² dose was selected for further experiments (publication I, figure 1D). The analysis of secreted hyaluronan from different time points (4, 24 and 48 h) showed a decrease at 24 h in UVB-treated cells (publication I, figure 1E). Pericellular hyaluronan content stayed at the same level throughout the observation period and was not influenced by UVB (publication I, figure 1F).

5.2.2 Pericellular hyaluronan coat in UV-response is produced by HAS2
To study the expression of HASes, RNA samples were collected from melanocyte cultures exposed to UVB ± Streptomyces hyaluronidase at 4, 24 and 48 h time points. Melanocytes were found to express all HASes and UVB-exposure ± hyaluronidase-treatment showed constant upregulation of HAS1 at all time points (publication I, figure 2A). In contrast, HAS2 and HAS3...
showed a strong downregulation at 4 h of 95% and 70%, respectively, in UVB and hyaluronidase treated melanocytes (publication I, figure 2B and C). HAS2 expression returned to control levels at 24 h post UVB-exposure, whereas hyaluronidase treatment with UVB further upregulated HAS2 expression by 1.5-fold at 48 h (publication I, figure 2B). HAS3 seemed to recover much slower; with expression reaching control levels at 48 h after irradiation in cultures treated with UVB-only, or in combination with hyaluronidase (publication I, figure 2C). A similar expression trend to HAS3 was detected in CD44. UVB-exposure with or without hyaluronidase-treatment reduced CD44 expression by 50% at 4 h, and the expression returned close to control levels at 24 h (publication I, figure 2D). HYAL-2 expression was downregulated by 35% at 4 h, but its recovery was more rapid, exceeding the untreated control at 24 h with different treatments (publication I, figure 2E).

To study which of the main hyaluronan synthase is responsible for producing the pericellular hyaluronan coat, siRNA silencing for the HASes and CD44 was used. Secreted and trypsin released (pericellular) hyaluronan was measured with a sandwich type hyaluronan-ELSA. This test showed that silencing of HAS2 decreased the amount of secreted hyaluronan in both non-irradiated and irradiated cultures by over 70% compared to non-irradiated, control siRNA treated cultures. HAS3 or CD44 silencing did not influence the release of hyaluronan to the culture medium while HAS1 silencing paradoxically increased it. In line with the findings in the figure 1, UVB reduced hyaluronan secretion by 50% in control siRNA-treated cultures (publication I, figure 1E and 2F). In cultures unexposed to UVB, silencing HASes or CD44 failed to significantly influence pericellular hyaluronan content compared to control siRNA-treated cultures (publication I, figure 2G). UVB alone did not influence pericellular hyaluronan content, whereas in UVB-exposed cells, HAS2 silencing caused over a 45% reduction compared to control siRNA treated cultures (publication I, figure 2G). Silencing HAS3 did not show any changes and HAS1 or CD44 silencing caused a 45% increase, respectively (publication I, figure 2G). These results indicate that HAS2 is the main enzyme producing the secreted hyaluronan in primary melanocytes. Furthermore, it also suggests UVB may activate HAS2 at the plasma membrane to produce pericellular hyaluronan; indeed, silencing is able to prevent the pericellular hyaluronan coat formation after UV-irradiation (publication I, figure 2F and G). The results show that UVB strongly affects HAS expression and thereby the amount of secreted hyaluronan. siRNA experiments indicated that the maintenance of the pericellular coat after the UVB was also mainly dependent on HAS2.

Live cell imaging revealed that the pericellular hyaluronan coat is rapidly, within 10 min, degraded by the Streptomyces hyaluronidase (1 TRU/ml). Also, restoration of the hyaluronan coat was surprisingly fast. Thus, a thick hyaluronan coat already surrounded the melanocytes 40 min after the digestion medium was changed to fresh medium lacking the enzyme, (publication I, figure 4A-C). This result indicates that hyaluronan synthesis is rapidly produced back at the plasma membrane, after the coat has been removed, for example with Streptomyces hyaluronidase.

5.2.3 Fragmentation of pericellular hyaluronan coat promotes inflammatory reaction in UVB-exposed melanocytes

Since UVB is known to induce an inflammatory reaction in skin, we used a cytokine array to study which inflammatory mediators are upregulated after UVB-exposure and how hyaluronidase treatment and thus the fragmentation of hyaluronan alters this reaction. The cytokine array revealed a strong upregulation of cytokine IL-6 and chemokines IL-8 and CXCL-1 in UVB treated melanocytes. The upregulation of these inflammatory mediators was even further heightened when UVB-exposed cells were treated with hyaluronidase. Interestingly, chemokine CXCL-10 secretion was induced only when hyaluronidase digestion was combined with UVB-exposure (publication I, figure 3A and B). The mRNA expression of IL-6, IL-8, CXCL-1 and CXCL-10 was also confirmed with quantitative qRT-PCR at 24 h. qRT-PCR results
showed a 10-fold and 27-fold increase in the expression of IL-6 and IL-8, and a more modest 2.9-fold and 5-fold increase in the expression of CXCL-1 and CXCL-10 in UVB-only exposed cells. Hyaluronidase digestion combined with UVB-irradiation more than doubled the effect of UVB on the expression of IL-6, IL-8, and CXCL-1 and increased the expression of CXCL-10 over 150-fold compared to the untreated control (publication I, figure 3C-F). *Streptomyces* hyaluronidase treatment alone slightly upregulated IL-8 and CXCL-1 expression by 2-fold but did not influence the other genes studied (publication I, figure 3E). This result indicates that hyaluronan degradation products and UVB increase the inflammatory response.

To further confirm that hyaluronan is involved in the *Streptomyces* hyaluronidase induced inflammatory reaction, the main hyaluronan synthase, HAS2, was silenced with siRNA in melanocytes. HAS2 silenced cells were not able to mediate inflammatory reaction after UVB or UVB with hyaluronidase as strong as control siRNA exposed cultures. The expression of IL-6, IL-8 and CXCL-1 showed a reduced trend by 40%, 40% and 20% in HAS2 silenced UVB-exposed melanocytes, respectively. HAS2 silenced UVB and hyaluronidase -treated melanocytes showed 60%, 55%, 40% and 25% reduction in IL-6, IL-8, CXCL-1 and CXCL-10 expression, respectively, compared to the same treatments in control siRNA melanocytes (publication I, figure 4D-G). This difference is probably due to a reduced amount of secreted and pericellular hyaluronan as shown in the hyaluronan coat experiments. HAS2 is the main synthase in melanocytes producing the secreted hyaluronan and restoring the pericellular hyaluronan coat after UVB. This HAS2 produced hyaluronan is the source for hyaluronidase to generate hyaluronan fragments and strengthen the inflammatory response. These results further reinforce the hypothesis that UVB-exposure, combined with the fragmentation of the pericellular hyaluronan coat by hyaluronidase, mediates the increased inflammatory response.

### 5.2.4 TLR-4 mediates hyaluronan fragment-induced cytokine and chemokine expression

To study which receptor is activated after UVB alone or with hyaluronidase -treatment, siRNA against CD44 and an inhibitor of TLR-4 (TAK-242) were used. Silencing CD44 only moderately reduced the expression of IL-6, IL-8 or CXCL-1 in UVB treated melanocytes, compared to the same treatments in control siRNA cultures. Instead, CD44 silencing increased the expression of CXCL-10 by 370% as well as modestly upregulated the expression of IL-8 and CXCL-1 in UVB and hyaluronidase treated melanocytes, whereas the expression of IL-6 was in similar level than in UVB and hyaluronidase treated control siRNA melanocytes (publication I, figure 4H-K). Using an inhibitor against TLR-4 receptor (10 μM, TAK-242), the activation of cytokine and chemokine expressions were significantly reduced. In the UVB-response, the TLR-4 inhibitor slightly reduced the expression of IL-6, of IL-8, of CXCL-1 and of CXCL-10. Similarly, in the UVB with *Streptomyces* hyaluronidase treated melanocytes, the TLR-4 inhibitor almost totally blocked the expression of IL-6, CXCL-1 and CXCL-10 and reduced the expression of IL-8 by 80% (publication I, figure 5A-D). The inhibitor of NF-κB subunit p65 nuclear localization (5 μM, JSH-23) was also used; this showed similar results as the TLR-4 inhibitor. NF-κB inhibition was able to reduce the expression of IL-6 (80%), IL-8 (50%), CXCL-1 (80%) and CXCL-10 (85%) in UVB-irradiated cells. However, the strongest response was for UVB and *Streptomyces* hyaluronidase treated cells which reduced expression of IL-6 by 85%, IL-8 by 80%, CXCL-1 by 90% and CXCL-10 by 95% (publication I, figure 5E-H). This result suggests a role for NF-κB downstream of TLR-4 in the UVB response with or without hyaluronidase.

### 5.2.5 The pericellular hyaluronan digestion in UVB response induces strong p38 and AKT activation

To study the signaling cascades after UVB alone or with hyaluronidase treatment, phosphorylation of p38 and AKT was studied. The phosphorylation of p38 was already upregulated by 5-fold after 30 min of UVB-exposure and stayed stably upregulated at 4 (10-fold) and 24 (6-fold) h post irradiation. *Streptomyces* hyaluronidase treatment together with UVB also showed a strong upregulation of p38, but in similar intensity than UVB alone (publication
The AKT activation induced by UVB alone was more modest than p38 activation, only reaching significance at 4 h. However, in contrast to p38, a combination of UVB and hyaluronidase showed intensified phosphorylation of AKT, compared to UVB-only. This increase was already 25% at 30 min post irradiation, and reached 150% at 4 h, compared to the untreated control, and 60% compared to UVB-only (publication I, figure 6C and D).

The function of activated p38 and AKT signaling on melanocytes cytokine and chemokine expression was studied using inhibitors against the phosphorylation of p38 (10 µM, BIRB796) and AKT (5 µM, VII). The inhibitor against p38-mediated signaling was able to reduce IL-6 and IL-8 expression by 65% and 55% in UVB-exposed cells, whereas no change in the expression of CXCL-1 and CXCL-10 was detected. Similarly, the p38 inhibitor was able to reduce the expression of IL-6 by 80%, IL-8 by 70%, CXCL-1 by 30% and CXCL-10 by 95% in UVB-exposed cells treated with hyaluronidase (publication I, figure 6E-H). Inhibiting AKT signaling was not able to inhibit the cytokine and chemokine expression induced by UVB alone, or UVB together with Streptomyces hyaluronidase, except for the expression of CXCL-10 induced by the combination treatment, which was reduced by 70% (publication I, figure 6H). This inhibitor data indicates that p38 is involved in the inflammatory reaction with UVB alone or together with hyaluronidase whereas AKT signaling possibly mediates other pathways such as cell survival.

**Figure 7.** Summary of the results from publication I. Melanocytes contain a thick pericellular hyaluronan coat mainly produced by HAS2. UVB-exposure induces TLR-4 receptor-mediated signaling leading to NF-κB activation and proinflammatory cytokine and chemokine expression in melanocytes. Pericellular hyaluronan coat fragmentation by Streptomyces hyaluronidase treatment combined to UVB-exposure further intensifies this inflammatory reaction. Moreover, melanocytes exposed to UVB together with hyaluronidase activate AKT signaling.

5.3 HAS3 OVEREXPRESSION IN METASTATIC MELANOMA CELL LINE

5.3.1 HAS3 overexpression increases hyaluronan secretion and HAS3-positive cell protrusions

To study the effect of HAS3 overexpression/increased hyaluronan production in a metastatic melanoma cell line, a doxycycline inducible EGFP-HAS3-MV3 cell line was generated using
lentiviral transduction. The efficacy of overexpression of HAS3 to hyaluronan production was studied with a dose-response using different doxycycline doses ranging between 0−1 µg/ml. Culture media were collected from cells after 48 h and analyzed using a sandwich type hyaluronan-ELSA. This test showed that doxycycline strongly induced hyaluronan production in HAS3-MV3 cells (publication II, figure 1A). For further studies, 0.1 µg/ml of doxycycline dose was selected because this induced hyaluronan synthesis and HAS3 expression significantly, but not too much. qRT-PCR analysis verified the sandwich type hyaluronan-ELSA results. Doxycycline strongly induced HAS3 expression compared to uninduced HAS3-MV3 cells. Induced HAS3 expression was coupled with increased HAS1 expression, but no changes in HAS2 expression were detected (publication II, figure 1C). A hyaluronan molecular mass assay demonstrated that HAS3 overexpression increased high molecular weight hyaluronan production (publication II, figure 1B). Doxycycline itself did not induce any of the three HASes nor hyaluronan synthesis in the parental MV3 cells.

Doxycycline induced EGFP-HAS3 production was fast and EGFP-HAS3 was visible already after 2 h of doxycycline induction in live cells when studied with confocal microscopy. The signal for EGFP-HAS3 was detected intracellularly at the Golgi area and at the plasma membrane. Hyaluronan secretion was also accelerated at this time point and the pericellular hyaluronan coat was thicker compared to uninduced cells (publication II, figure 2A). After 24 h induction, EGFP-HAS3 induced cells showed typical microvilli and EGFP-HAS3-positive protrusions pointing upwards from the plasma membrane in live cells (publication II, figure 2C). The pericellular hyaluronan coat was thick and intensely stained for hyaluronan compared to the uninduced cells (publication II, figure 2B). Hylanuronan was also stained with CD44 in 4% PFA fixed cells. Typical EGFP-HAS3 protrusions seen in doxycycline-induced live cells collapsed during the fixation process (publication II, figure 3A). The EGFP-HAS3 signal was found at the Golgi area and at the tip of these protrusions. Equally intense staining of CD44 was detected in uninduced and induced cells at the plasma membrane. The numerous protrusions in EGFP-HAS3 induced cells were also CD44 positive (publication II, figure 3C). In doxycycline-induced cells, hyaluronan was spread around the cells and accumulated apically in the microvilli as well as basally below the cells. Uninduced cells showed lower hyaluronan staining; there was less hyaluronan on the apical side of the plasma membrane as well as on the basal side (publication II, figure 3B). The results indicate that doxycycline induction activates HAS3 expression and hence it rapidly starts hyaluronan production at the plasma membrane, and later on, typical HAS3-positive microvilli protrusions.

5.3.2 HAS3 overexpression reduces cell proliferation arresting the cells in G1/G0 phase
To study the effect of EGFP-HAS3 overexpression and elevated hyaluronan production on cell proliferation, cells were plated on 24-well plates, induced and counted daily. Induced EGFP-HAS3 cells showed reduced cell proliferation compared to uninduced cells. Cell cycle analysis revealed that induction of EGFP-HAS3 arrests the cells in the G1/G0 phase and therefore reduces the proportion of cells in the S and G2/M phases (publication II, figure 4C). These experiments were also performed in the parental MV3 cells treated with similar concentrations of doxycycline, which showed no changes in proliferation, or cell cycle phases. Hylanuronan hexasaccharides (HA6), which are known to bind CD44 and thus block hyaluronan binding, were combined with or without doxycycline induction to study whether the proliferative signal is mediated via CD44. Uninduced cells and HA6 treated cells showed similar growth curves. Doxycycline-induced and doxycycline combined with HA6 both showed decreased cell proliferation, even though HA6 in induced cells showed minor reversion in cell number (publication II, figure 4A). To study the influence of pericellular hyaluronan on cell proliferation, pericellular hyaluronan was degraded with Streptomyces hyaluronidase in doxycycline-induced cells. Hylanuronan degradation restored the proliferative capacity of doxycycline treated cells to control levels (publication II, figure 4B) indicating that enhanced hyaluronan synthesis and accumulation inhibit EGFP-HAS3 induced melanoma cell growth.
5.3.3 HAS3 overexpression reduces cell migration and adhesion

Since hyaluronan has been reported to affect cell migration and invasion in several cell types, we also studied the influence of EGFP-HAS3 on cell locomotion and adhesion. Cell migration was studied using a scratch wound assay, taking images at 0, 6, 12 and 24 h intervals (publication II, figure 5A) and tracking single cell random migration for 6 h (publication II, figure 5B). Both migration assays showed that doxycycline-induced EGFP-HAS3 overexpression reduced cell migration compared to uninduced cells. The effect of EGFP-HAS3 overexpression on cell migration was more apparent in a random cell migration assay compared to scratch wound assay (publication II, figure 5B). Similar to the proliferation assay, HA6 and Streptomyces hyaluronidase were combined with or without doxycycline in migration experiments. EGFP-HAS3 overexpression alone or together with HA6, showed reduced migration at 12 h, as well as at 24 h. However, migration at 24 h in EGFP-HAS3 overexpressing cells together with HA6 treatment was slightly increased compared to EGFP-HAS3 overexpressing cells alone; this change was statistically significant. HA6 treatment in uninduced control cells showed the fastest migration capacity (publication II, figure 5C). Streptomyces hyaluronidase -treatment annulled the effect of EGFP-HAS3 overexpression on MV3 cell migration. The migratory capacity of EGFP-HAS3 induced cells treated with Streptomyces hyaluronidase was equal to control cells (publication II, figure 5D). This result suggests a hyaluronan-CD44 interaction in the migration of induced EGFP-HAS3 melanoma cells.

To study whether the increased pericellular hyaluronan coat around the cells affects cell adhesion, cells were plated on collagen type I (0.1 mg/ml) coated wells. Cells were induced with doxycycline 48 h prior to an adhesion assay. Doxycycline induced EGFP-HAS3 overexpressing cells showed reduced adhesion capacity compared to uninduced cells; the stronger the HAS3 induction, the lower the adhesion. Streptomyces hyaluronidase -treatment 20 min prior to plating the cells to the adhesion assay did not alter the result (publication II, figure 5E), indicating that it is not just the pericellular hyaluronan which results in reduced cell adhesion in EGFP-HAS3 overexpressing MV3 cells. Focal adhesion staining using a vinculin antibody in fixed (4% PFA) cells showed that EGFP-HAS3 overexpression reduced the number of focal adhesions at the cell periphery compared to uninduced cells (publication II, figure 5F). This result demonstrates that either induced EGFP-HAS3 or produced hyaluronan mediates intracellular signaling cascades involved in the formation of adhesion complexes, rather than the excess hyaluronan itself.

5.3.4 HAS3 overexpression reduces the activation of growth signal pathways in melanoma cells

To study which phosphokinase signaling pathways are activated after induction of EGFP-HAS3 overexpression, samples were collected at 2 and 6 h after starting the induction and a commercial phosphokinase array was carried out. Phosphokinase array analysis showed reduced phosphorylation of Src-family kinases (Src, Lyn, Fyn, Yes and Fgr) in EGFP-HAS3 overexpressing cells after 2 h of induction (publication II, figure 6A). After 6 h induction, EGFP-HAS3 overexpressing cells showed a strong activation of STAT1 (2-fold) and STAT4 (1.5-fold) (publication II, figure 6B). The phosphorylation of p38α, MEK1/2 and MSK1/2 was decreased over time from 2 h to 6 h in EGFP-HAS3 overexpressing cells (publication II, figure 6C). Reduced pERK1/2 signaling was later verified from the protein samples in western blotting. Protein samples collected from doxycycline induced parental MV3 cells did not show similar reduced pERK1/2 signaling as EGFP-HAS3 cells, indicating that doxycycline alone is not responsible for reduced pERK1/2 activation. Moreover, the activation of transcription factor NF-κB expression was reduced by 20% in EGFP-HAS3 overexpressing cells, compared to uninduced cells (publication II, figure 6D). Analysis of the signaling proteins involved in the
cell division supports our data that EGFP-HAS3 and increased hyaluronan synthesis reduces cell proliferation.

Figure 8. Summary of the results from the publication II. MV3 melanoma cells were transduced with lentivirus to dose dependently overexpress HAS3. HAS3 overexpression and hence increased hyaluronan production reduced melanoma cell proliferation and arrested the cells at the G1/G0 phase. Decreased proliferation was coupled with reduced ERK1/2 signaling activity. Increased hyaluronan production by HAS3 also decreased melanoma cell migration and adhesion.

5.4 THE EFFECT OF MELANOMA CELLS SECRETED FACTORS ON STROMAL FIBROBLASTS

5.4.1 Melanoma cells secreted factors induce hyaluronan synthesis in dermal fibroblasts via HAS2

Growth medium containing 1% FBS was collected from the metastatic C8161 melanoma cell line after 48 h of growth. This was first filtered in a 0.2 µm filter to remove cell debris, then concentrated with 30 kDa cut-off filters 40 times and finally diluted with fresh medium in 1:10. Same basal medium was concentrated in a similar manner and used as a control medium. Fibroblasts were treated with the control and melanoma cell conditioned medium (CM) for 24 h and its effect on hyaluronan secretion was measured with a sandwich type hyaluronan-ELSA. Melanoma cell CM highly increased fibroblast hyaluronan synthesis compared to concentrated or unconcentrated control medium or collected growth medium itself without concentration and dilution (publication II, figure 1A). Size exclusion chromatography revealed that CM induced the production of high molecular weight hyaluronan as well as hyaluronan of lower molecular size (publication III, figure 1B).

Melanoma cell CM induced the expression of HAS2 by 20-fold, but not the expression of other HASes, CD44 or HYAL-2 (publication III, figure 1C). Silencing of HAS2 with siRNA transfection was able to reverse the effect of melanoma cell CM, indicating that HAS2 is the responsible enzyme for hyaluronan production in CM-treated fibroblasts. HAS2 staining for light microscopy also showed a strong staining intensity when fibroblasts were treated with CM compared to control (publication III, figure 1E and F). HAS2 silencing also decreased hyaluronan secretion by 50% in control medium treated fibroblasts, indicating that HAS2 is the
main enzyme in fibroblasts producing hyaluronan under basal conditions. HAS3 siRNA did not show any effect on fibroblasts hyaluronan secretion either in basal or in CM-induced cells (publication III, figure 1D). These results indicate that melanoma cells secrete factors stimulate fibroblast hyaluronan synthesis by upregulating HAS2 expression.

5.4.2 Conditioned medium induces hyaluronan coat formation and morphological changes in fibroblasts
Confocal microscopy reproduced the results from light microscope; CM-treated fibroblasts showed intense hyaluronan staining, both pericellular and intracellular hyaluronan were increased (publication III, figure 2A and B). Melanoma cell CM treatment had no effect on fibroblast CD44 expression or localization (publication III, figure 2C and D). Live cell imaging with confocal microscopy using fHABC showed that the pericellular hyaluronan coat was thicker and more intense in CM-treated fibroblasts compared to control cells. CM-treated fibroblasts also formed hyaluronan positive cable-like structures, which were not seen in control cultures (publication III, figure 2D, G and H). These kinds of hyaluronan cables are seen during inflammation (de La Motte et al., 1999, Jokela et al., 2008b) but could also be present in tumor stroma where they could attract and bind inflammatory cells to the tumor area (Itano, Zhuo & Kimata, 2008). To study the increase of intracellular hyaluronan, extracellular hyaluronan was degraded with Streptomyces hyaluronidase before staining. CM-treatment also increased the accumulation of intracellular hyaluronan which was mainly localized around the nucleus (publication III, figure 2I and J). The accumulation of intracellular hyaluronan is also seen in other cell types with upregulated hyaluronan synthesis (Karvinen et al., 2003b, Pienimäki et al., 2001, Pasonen-Seppänen et al., 2003).

Upregulation of HAS2 in CM treated fibroblasts correlated with altered morphology. After CM treatment, fibroblasts were more elongated and contained long and thin plasma membrane protrusions. These protrusions were positive for actin and CD44, as studied with confocal microscopy (publication III, figure 5E and F). The axial ratio was measured to quantify this morphological change which was 2-fold in CM-treated fibroblasts compared to control fibroblasts (publication III, figure 5G). Moreover, CM and control medium treated fibroblasts were processed for scanning electron microscopy to examine the ultrastructure of these cells in more detail. Scanning electron microscopy confirmed altered morphology observed with light microscopy (publication III, figure 5A-D). This could indicate that CM-treatment transforms these fibroblasts towards more myofibroblasts-like cells.

5.4.3 PDGF-receptor mediates HAS2 upregulation via PI3K-AKT and p38 signaling
The influence of CM-treatment on the activation of receptor tyrosine kinases was analyzed with a commercial phospho-RTK array. This array indicated that CM activates EGFR and PDGFRα and PDGFRβ receptors within 15 min. Control treatment did not activate any of the studied RTKs (publication III, figure 3A). To study which intracellular phosphokinase pathways are activated with CM-treatment, samples for a commercial phosphokinase array were collected after 30 min and 2 h CM-treatment. This showed that CM strongly induces the activation of p38, JNK pan, AKT (S473), AKT (T308), CREB, HSP27, STAT3 and cJUN, compared to control treatment (publication III, figure 3B and C). The phosphorylation of pERK1/2 and p38 were also confirmed in Western Blot, after treating the fibroblasts with either control medium or CM for 24 h (publication III, figure 3D).

To study which signaling routes mediate the CM response from PDGFR to HAS2 upregulation and hence increased hyaluronan synthesis, inhibitors against PI3K, AKT, p38, MEK, PDGFR and EGFR were used together with CM-treatment. Inhibitors were added 30 min prior to CM-treatment and hyaluronan secretion in culture medium was analyzed after 24 h of treatment. This experiment showed that AKT and p38 inhibitors reduced the effects of CM by 50%, but the strongest effect was obtained with a PDGFR inhibitor, which was able to totally
block the effect of CM on hyaluronan secretion to control levels. The EGFR inhibitor and MEK inhibitor only showed a 25% reduction in hyaluronan synthesis in CM treated fibroblasts (publication III, figure 4A).

siRNAs against PDGFRα/β were used to verify the effect of PDGFR in the melanoma CM-induced hyaluronan response and gene expression. PDGFR silencing significantly reduced melanoma CM-induced HAS2 expression (publication III, figure 4B). Moreover, the PDGFR inhibitor used was also able to reverse the effect of melanoma CM on AKT activation in fibroblasts (publication III, figure 3E) confirming the signaling route that melanoma cell CM -treatment induces.

5.4.4 Conditioned medium increases MMP-1 and MMP-9 expression in fibroblasts and their invasion

The elongated morphology with several protrusions suggested that CM treated fibroblasts are active and motile. Their potential to invade was studied with a collagen-Cultrex invasion assay developed in our lab. In this assay, collagen-Cultrex 1:1 gel is cast on the top of confluent cell culture. Thereafter, cell invasion is followed for 24 to 48 h, fixed and stained for actin and examined with confocal microscopy. CM-treatment increased fibroblast invasion by 40%, which was prevented either with PDGFR inhibitor treatment prior to CM-treatment, siRNAs against PDGFRα/β or siRNA against HAS2. HAS2 or PDGFRα/β silencing reduced the invasion of fibroblasts below control levels, even when the cells were treated with CM (publication III, figure 7A-D). These results suggest that PDGFRα/β induced HAS2 expression, and hence hyaluronan synthesis, is essential for fibroblasts invasion.

Since MMPs are important for cell invasion, we studied the expression of MMP-1, MMP-2, MMP-9 and MMP-14 with qRT-PCR. The CM treatment induced greatly MMP-1 (340-fold), MMP-9 (100-fold) and MMP-14 (2-fold) expression. Silencing PDGFRα/β using a siRNA was able to reduce the expression of MMP-9, but not MMP-1, MMP-2 or MMP-14 (publication III, figure 7E). This indicates that CM-treatment induced MMP-9 as a mediator for increased fibroblasts invasion.

In addition to invasion, the influence of CM on cell proliferation was examined by counting the treated cells for 3 days. Fibroblasts treated with CM proliferated faster than control cells (publication III, figure 6) indicating that the mediators in the CM also induce fibroblast cell division.
Figure 9. Summary of the results from the publication III. Conditioned media (CM) collected from C8161 melanoma cells induced PDGFRα and -β activation leading to AKT and p38 phosphorylation and increased HAS2 expression and hyaluronan synthesis in dermal fibroblasts. CM-induced fibroblasts were more elongated, invasive and the expression of MMP-1, MMP-2, MMP-9 and MMP-14 was increased. Inhibiting either PDGFRα and -β activation or HAS2 expression these changes after CM-treatment were prevented.
Discussion

Environmental factors, such as UV radiation, and mutations in genes involved in the cell growth pathways, such as BRAF or AKT, contribute to changes in melanocytes and their transformation. However, the triggering mechanism is unidentified and obscure. Hyaluronan, a pericellular and an extracellular sugar molecule of high molecular weight, maintains the homeostatic balance in the extracellular milieu and responds to cues from the microenvironment. The role of hyaluronan in primary melanocytes and in melanomagenesis is unknown and has not been studied before.

The purpose of this doctoral thesis was to study and determine the role of hyaluronan in the early phases in primary melanocytes towards dysplastic cells and melanoma. We also wanted to discover the effect of hyaluronan in metastatic melanoma cells and in the tumor microenvironment, especially between the interactions of melanoma cells and stromal fibroblasts.

6.1 HYALURONAN IN THE EARLY STAGES OF MELANOMA GENESIS

6.1.1 The diversity of hyaluronan metabolism in primary cells

Our study provides novel data on hyaluronan metabolism in primary melanocytes and this has not been investigated before. Our results indicate that melanocytes have a thick pericellular hyaluronan coat and secrete high amounts of hyaluronan in the culture medium, similar to many other primary cells such as skin fibroblasts (Wang et al., 2014) and primary rat mesothelial cells (Koistinen et al., 2016) or human bone marrow-derived mesenchymal stem cells (Qu et al., 2014). However, this result is in contrast to primary epithelial cells, such as keratinocytes (Pasonen-Seppänen et al., 2012). HAS2 was found to be the main synthase producing hyaluronan released in the culture medium and retained in the pericellular matrix in melanocytes, which has also been shown in fibroblasts (Wang et al., 2014), mesothelial cells (Koistinen et al., 2016), keratinocytes (Pasonen-Seppänen et al., 2012) and mouse mesenchymal tissue during embryonic development (Törnönen et al., 2014).

Melanocytes were relatively tolerant of UVB and cell numbers were not affected even with higher doses (70 mJ/cm²). This is contrast to cells such as epidermal keratinocytes, which have a smaller pericellular hyaluronan coat (Pienimäki et al., 2001). In keratinocytes, even low doses of UVB reduce cell viability (Rauhala et al., 2013). The function of the pericellular hyaluronan coat in different type of external stress, such as UVR, is still under investigation but Pauloin et al. (2009) suggested that hyaluronan protects corneal epithelial cells against UVB-induced apoptosis (Pauloin et al., 2009). Similar results were reported by Wang et al. (2014), showing that pericellular hyaluronan protected dermal fibroblasts from UVB-induced apoptosis (Wang et al., 2014) supporting the hypothesis that a thick pericellular hyaluronan coat may be protective against external threats, such as UVB. In line with this, in melanocytes the thick pericellular hyaluronan coat could provide a similar shield, explaining the high tolerance for UVB. However, in our model, HAS2 silencing did not affect the viability of the melanocytes even though it prevented the restoration of the pericellular hyaluronan coat in the UVB-response; these results are opposite to dermal fibroblasts (Wang et al., 2014). While, apoptosis markers were not studied, visual exploration of the cells indicated normal, viable morphology. This may be explained by its more modest effect on pericellular hyaluronan, despite a marked inhibition of hyaluronan synthesis.
The pericellular hyaluronan coat is often bound to the plasma membrane via its receptor CD44, such as in chondrocytes (Knudson, 1993) and keratinocytes (Pasonen-Seppänen et al., 2012). Our results in primary melanocytes show that CD44 silencing did not affect the amount of secreted hyaluronan, nor the pericellular hyaluronan; more likely it increased the pericellular hyaluronan coat in the UVB response. These results indicate that hyaluronan is not mainly bound to its plasma membrane receptor CD44, even though it can mediate signals through it.

6.1.2 UVB exposure induces changes on hyaluronan related genes expression

The reported responses for UVB differ quite substantially which is probably due to the different characteristics of different cell types (like the thickness of the hyaluronan coat) but also due to different sources of UVB, the wavelength range, the dose and the exposure surrounding; all of these factors make it difficult to compare the published results. Furthermore, the UVB-mediated effects can be direct or indirect via mediators such as ROS (Hašová et al., 2011, Pauloin et al., 2009, Soltes et al., 2006).

Our data showed that a UVB dose of 30 mJ/cm² decreased the amount of secreted hyaluronan in melanocytes, whereas the pericellular hyaluronan was not affected after a single dose. Digesting the pericellular hyaluronan coat with Streptomyces hyaluronidase, which generates a pool of hyaluronan fragments of different size (Price et al., 1997), was studied in melanocytes using two different hyaluronan-ELSA methods; a competitive hyaluronan-ELSA which is more sensitive to LMW hyaluronan (Hämäläinen et al., 2018) and a sandwich type hyaluronan-ELSA (Hiltunen et al., 2002). Comparing these two methods, the competitive hyaluronan-ELSA showed that a higher portion of pericellular hyaluronan remained at the plasma membrane even though treated with hyaluronidase. In the UVB-response, this pericellular portion in digested cultures attained close to the level of UVB alone at 24 h, indicating active hyaluronan synthesis restores the pericellular coats in melanocytes. Furthermore, both in the fibroblasts (Wang et al., 2014) and here in melanocytes, digestion of the hyaluronan prior to UVB with hyaluronidase, did not influence the cell viability. Actually, our data showed that the enzyme leaves considerable amounts of hyaluronan on the cell surface. It remains to be shown, whether such a coat consisting of hyaluronan stubs could protect the cells.

UVB-exposure is shown to increase hyaluronan synthesis via sequential induction of HAS1−3 expression in rat epidermal keratinocytes (Rauhala et al., 2013). HAS1 expression in melanocytes showed a similar trend after UVB-exposure, as in keratinocytes (Rauhala et al., 2013) and skin fibroblasts (Wang et al., 2014). HAS2 and HAS3, on the other hand, were downregulated initially. HAS3 recovery to unexposed levels was much slower compared to HAS2, also pointing towards similar results observed in skin fibroblasts (Wang et al., 2014) and regards to HAS2 in human epidermal keratinocytes (Hašová et al., 2011). The most interesting result was that hyaluronan digestion with Streptomyces hyaluronidase together with UVB-exposure increased HAS2 expression, which was significantly upregulated compared to UVB-only after 48 h. Alongside this, the expressions of HAS1 and HAS3 were not influenced. The secreted hyaluronan was clearly decreased in UVB-exposed cultures, whereas the pericellular hyaluronan stayed at the same level as control cultures. When HAS1−3 was silenced with siRNA transfection, HAS1 and HAS3 silencing had no effect on reducing the secreted hyaluronan or the pericellular hyaluronan. HAS2 silencing indicated that HAS2 is responsible for the production of secreted hyaluronan to the culture medium, as well as the pericellular hyaluronan after UVB. Although HAS1 expression was upregulated after UVB, its silencing unexpectedly increased the pericellular hyaluronan, in contrast to reducing the production of hyaluronan in the medium or in the pericellular matrix.

As previously reported in human epidermal keratinocytes (Hašová et al., 2011) we also observed that UVB increases the secretion of IL-6 and IL-8 together with CXCL-1. Silencing HAS2, the main synthase producing hyaluronan in melanocytes, reduced the effect of UVB on the expression of cytokine and chemokines studied. This novel finding indicates that
hyaluronan synthesized by HAS2 may have an effect in the first line reaction after UVB and in the production of the proinflammatory factors attracting immune cells.

6.1.3 Hyaluronan degradation accelerates pronounced inflammation during UVB-exposure

UV directly, or indirectly via free radicals have been shown to cause hyaluronan fragmentation (Deguine et al., 1997, Esser et al., 2012, Kennett, Davies, 2007, Yu et al., 2011a). Monzon et al. (2010) showed that in airways free radicals lead to formation of hyaluronan fragments of 75 kDa in size. Somewhat surprisingly our results did not show any formation of hyaluronan fragments neither in the culture media nor in the pericellular coats in melanocytes (24 h) after UVB (data not shown). Similarly, no signs of hyaluronan fragmentation were observed when keratinocyte monolayers were exposed to UVB (Rauhala et al., 2013), alike to the findings of Kakizaki et al. (2008). Although we do not know the reason for this discrepancy, it is possible that a single UVB-exposure is not enough to induce hyaluronan depolymerization and chronic UVB irradiation is necessary. However, fragments shorter than the detection limit of the assay (10 kDa), if generated, will not be detected. The other alternative explanation is that fragmentation is rapid and hyaluronan fragments are quickly taken up into the cells or hyaluronan degradation may require involvement of other cell types in a co-culture manner, such as in intact skin, where hyaluronan fragments were detected 24 h after irradiation (Averbeck et al., 2007). As it is known that externally added hyaluronidase increases hyaluronan fragmentation (Price et al., 1997), we wanted to stimulate a situation where this occurs like in intact skin (Averbeck et al., 2007). We combined hyaluronidase with UVB-exposure to mimic chronic UVB irradiation to create a situation where there is a pool of hyaluronan fragments available in the cell microenvironment. Although, hyaluronidase will degrade hyaluronan to short oligosaccharides (<10 kDa), which ROS are unlikely to generate, at first longer fragments are formed and the synthesis of new macromolecular hyaluronan supplies new material for their formation. Indeed, detectable levels of hyaluronan (>10 kDa) were present in the hyaluronidase treated melanocytes’ pericellular coats with or without UVB. Both the longer fragments and hyaluronan oligosaccharides (<10 kDa) are biologically active and have been shown to trigger inflammatory responses or induce angiogenesis although their effects may differ (Gao et al., 2008, Litwiniuk et al., 2016, Campo et al., 2013, Campo et al., 2010, Cui et al., 2009, Termeer et al., 2000, Tolg, Telmer & Turley, 2014). Interestingly, combining hyaluronan degradation with UVB-exposure led to a further elevation in the secretion and expression of proinflammatory mediators IL-6, IL-8, CXCL-1 and bringing up also the CXCL-10. However, whether combining HMW hyaluronan with UVB-exposure could reverse this proinflammatory reaction, was not studied.

To make sure that the stimulatory effect of Streptomyces hyaluronidase is not due to, for example LPS contamination in the preparation, similar to some earlier publications by others groups (Ebied, Lichtnekert & Anders, 2014, Dong et al., 2016), the enzyme was inactivated by boiling and its effects on inflammatory reaction were studied. Boiled hyaluronidase was not able to boost the inflammatory reaction above UVB-exposure, indicating that the stimulatory effect is due to active hyaluronan depolymerization, not LPS contamination (publication I, supplementary figure 1A). This result is in line with HAS2 silencing in UVB-exposed melanocytes. Silencing HAS2 abolished the stimulatory effect of hyaluronidase in UVB response further confirming the specificity of hyaluronidase and hyaluronan degradation products in the inflammatory response. Surprisingly, treating the cells prior to UVB with hyaluronidase, but not after, failed to induce IL-6 or IL-8 mRNA expression above the UVB-only treatment (publication I, supplementary figure 1B). These results indicate that hyaluronan synthesis and production of a continuous pool of active hyaluronan degradation products (fragments/oligosaccharides) are needed to induce proinflammatory IL-6, IL-8, CXCL-1 and CXCL10 mRNA expression and their secretion in UVB-irradiated cells.

As our data indicate, hyaluronidase treatment alone does not induce similar expression of proinflammatory reactions as was shown with oligosaccharides in melanoma (Voelcker et al.,
2008) or with LMW hyaluronan in breast cancer (Bourguignon et al., 2011), without UVB exposure. This result possibly indicates that, 1) UVB modifies these hyaluronan fragments/oligosaccharides to more reactive ones, or 2) induces different signaling pathways for yet unknown reasons, or 3) the preparations used in the previous studies were not pure. These results suggest a new relevance for hyaluronan fragmentation in the UVB response that needs to be further examined.

6.1.4 CD44 and TLR-4 receptor-mediated signaling in UVB-induced melanocytes
Hyaluronan can signal via receptors, including CD44, TLR-2 and -4 or RHAMM (Schwertfeger et al., 2015). High molecular weight hyaluronan is generally thought to maintain tissue homeostasis; its association with CD44 regulates, for example, keratinocyte differentiation in skin (Bourguignon et al., 2006) and maintains their normal physiology (Shatirishvili et al., 2016). On the other hand, UV-induced hyaluronan fragments mediate CD44-dependent signaling, resulting in an inflammatory response via NF-κB (Bourguignon, Bikle, 2015) and hyaluronan dodecasaccharides (HA12) induce CXCL-1 expression via CD44 (Takahashi et al., 2005). These findings are not supported by our data. Thus, CD44 silencing with siRNA slightly reduced the proinflammatory response induced by UVB alone whereas combining hyaluronidase with UVB-exposure CD44 silencing was not able to prevent the proinflammatory reaction. Instead, CD44 silencing notably increased the expression of CXCL-10 and modestly the expression of IL-8 indicating that CD44 was not anymore involved in the proinflammatory response when the pericellular hyaluronan coat was degraded. This could point towards a homeostatic nature of hyaluronan-CD44 interaction in primary melanocytes.

TLR-2 and -4 are shown to mediate signaling via hyaluronan oligosaccharides or low molecular weight hyaluronan (Campo et al., 2013) and to promote proinflammatory cytokine IL-6 (Bourguignon et al., 2011), chemokine IL-8 and MMP-2 secretion from cells via NF-κB activation (Voelcker et al., 2008). These findings are in line with our data. Indeed, blocking either TLR-4 or NK-κB was able to reduce the expression of IL-6 and IL-8 and almost prevent the expression of CXCL-1 or CXCL-10 when hyaluronidase was combined with UVB irradiation.

CD44 is decreased in metastatic melanoma samples (Siiskonen et al., 2013, Karjalainen et al., 2000) supporting the idea of a balancing effect for HMW hyaluronan-CD44 interactions to control the melanocyte homeostasis; this hypothesis is supported by our in vitro data. When the threshold is exceeded from HMW hyaluronan-CD44 signaling towards signaling via hyaluronan fragments-TLR-4, it could favor the inflammatory effect resulting in the formation of dysplastic melanocytes and malignant changes in the long term. Cutaneous malignant melanoma patient samples show positive immunostaining for TLR-4, which is associated with relapse and poor prognosis (Eiro et al., 2013). The same is also demonstrated from melanoma patient microarray data that shows melanoma tumor cells and infiltrating lymphocytes overexpress TLR-4 (Mittal et al., 2010). These studies support our hypothesis that hyaluronan fragments have an effect in TLR-4 mediated inflammation and melanomagenesis, but this should still be investigated in more detail in the future.

6.1.5 UVR induces activation of p38 and AKT signaling pathways
UVB-exposure can activate several signaling cascades including p38 in rat keratinocytes (Rauhala et al., 2013) and primary melanocytes (Terazawa, Imokawa, 2017) as well as the PI3K-AKT-mTOR cascade in mouse keratinocytes (Bridgeman et al., 2016). Mutations in the AKT signaling pathway are common in melanoma patients, where increased AKT signaling affects cell survival and inhibits apoptosis (Kang et al., 1999). The signaling cascades activated by hyaluronan or its fragments and UVB alone are overlapping (Rauhala et al., 2013, Hanabayashi et al., 2016, Schmitz et al., 2010, Bridgeman et al., 2016); however no studies on their joint effects on signaling have been published.

In line with Rauhala's studies (2013), we also observed a strong activation of p38 in UVB-irritated melanocytes already after 30 min. The phosphorylation of p38 stayed quite steady
during the whole observation period with both; UVB alone and together with *Streptomyces* hyaluronidase. AKT phosphorylation on the other hand, was activated only modestly with UVB, while *Streptomyces* hyaluronidase with UVB irradiation notably potentiated the effect of UVB to the phosphorylation of AKT. Inhibition of UVA-induced p38 signaling using L-carnitine reduced oxidative stress and the proinflammatory cytokine response of TNF-α, IL-6 and IL-1β (Salama et al., 2018). Similarly, EGb-761 antioxidant inhibited the UVB-induced activation of p38 and cJUN in human dermal fibroblasts and BALB/c mice skin and the expression of IL-1α, IL-1β and IL-6 (Chen et al., 2014). Using an inhibitor against p38 signaling in our *in vitro* UVB-model, we also observed a reduction in IL-6, IL-8, CXCL-1 and CXCL-10 expression compared to UVB alone. The effect was more evident when hyaluronidase was combined with UVB-exposure. However, inhibiting AKT in our studies did not lead to a reduction in the proinflammatory response either in UVB-exposed or UVB and hyaluronidase treated melanocytes. p38 and AKT pathways are possibly activated via different mechanisms and in a time-dependent manner in UV-response, as Zhang et al. suggested in human keratinocytes. UV induced rapid activation of p38, whereas ROS generated by UVB activated the AKT signaling and also prolonged the activation of p38 (Zhang et al., 2001). A similar mechanism for the activation of p38 and AKT could also be in UVB-exposed melanocytes. p38 activation seems to mediate the proinflammatory response, whereas AKT could potentiate other signaling pathways leading to cell survival. Evidently, many open questions remain to be investigated in the future, for example, the function of ROS and hyaluronidase in UVB response alone and together with hyaluronan fragmentation, as well as the downward signaling pathways, such as the interplay between p38 and AKT.

### 6.2 HYALURONAN METABOLISM IN THE METASTATIC STAGE OF MELANOMA

Siiskonen et al. have shown that patient samples from metastatic melanomas are almost negative for hyaluronan (2013). This decreased hyaluronan staining is probably due to reduced HAS1 and HAS2 expression combined with increased HYAL-2 expression (Siiskonen et al., 2013). It is also shown that reduced hyaluronan and HAS1/HAS2 expression are associated with poor patient survival (Karjalainen et al., 2000, Poukka et al., 2016). The effect of hyaluronan in tumors originating from stratified epithelia, such as the skin, differs from tumors originating from simple epithelia such as breast cancer (Tammi et al., 2008). To study the exact function of hyaluronan in melanoma, we generated HAS3 overexpressing melanoma cell lines. The lentivirally transduced inducible EGFP-HAS3-MV3 melanoma cell line produced high levels of hyaluronan. EGFP-HAS3 overexpression was already visible at the plasma membrane and Golgi as described previously (Rilla et al., 2005) at 2 h after induction. Similarly, the pericellular hyaluronan coat was visible and more apparent compared to uninduced cells, indicating a fast synthesis of hyaluronan from HAS3 overexpression. Longer HAS3 induction (24 h) resulted in a thick pericellular hyaluronan coat with numerous cell surface protrusions, similar that described in other HAS3 overexpressing cell types (Rilla et al., 2008, Kultti et al., 2006). Interestingly, similar kinds of cell surface protrusions have also been seen in cells which produce high levels of hyaluronan in a normal state, such as mesothelial and mesenchymal cells (Koistinen et al., 2016, Qu et al., 2014); this suggests that these plasma membrane protrusions are hyaluronan-producing ‘organelles’. Although, HAS3 has been reported to produce hyaluronan of lower molecular weight compared to HAS1 or HAS2 (Itano et al., 1999), in melanoma cells HAS3-induced hyaluronan was HMW in nature. High molecular weight hyaluronan prevails in healthy tissues and is more anti-inflammatory (Ruppert et al., 2014) whereas LMW hyaluronan is just the opposite, as shown in osteosarcoma (Tofuku et al., 2006). Possibly the HMW hyaluronan produced in HAS3 overexpressing cells also changes the melanoma cell behavior to be less inflammatory and aggressive.
There are no previous studies on the effect of endogenously produced hyaluronan on melanoma cell behavior, while studies of the effects of exogenously added hyaluronan of HMW, LMW, oligomers, or hyaluronidases and hyaluronan-receptor interaction have been performed, often showing variable results. For example exogenous HMW hyaluronan (1 MDa) have been shown to induce melanoma cell motility (Kim et al., 2008) whereas hyaluronan oligosaccharides (HA5) accelerate melanoma lymph node metastasis in mice (Du et al., 2016). Furthermore, hyaluronan oligosaccharides (HA4–6) induce TLR-4 - NF-κB mediated MMP-2 and IL-8 secretion in melanoma cells enhancing melanoma cell motility (Voelcker et al., 2008). There are also opposing results showing that hyaluronan oligomers (HA3–12) inhibit melanoma cells growth (Zeng et al., 1998). In melanoma xenografts, intratumoral hyaluronidase suppressed tumor growth (Guedan et al., 2010) and enhanced the efficacy of chemotherapy in melanoma mouse models (Spruss et al., 1995), whereas others have shown that hyaluronidase treatment can induce angiogenesis in melanoma in vivo (Liu et al., 1996). 4-MU reduces melanoma liver metastasis in mice by inhibiting hyaluronan synthase activity (Yoshihara et al., 2005) as well as melanoma cell adhesion and motility in vitro (Kudo et al., 2004). The different effects, especially in vivo models, could be due to the heterogeneity of the melanoma tumor microenvironment and the cells, which are exposed to exogenously added hyaluronan or oligosaccharides.

6.2.1 HAS3-produced hyaluronan decreases melanoma cell division
In prostate and pancreatic adenocarcinoma cancer cells, HAS3 overexpression has been shown to increase cell proliferation (Liu et al., 2001, Kultti et al., 2014) whereas our results indicate the opposite. In our results, HAS3 overexpressing MV3 melanoma cells showed decreased cell proliferation and hence increased hyaluronan synthesis arrested the melanoma cells at G1/G0 of cell cycle phase and lowered the portion of cells in S and G2/M phases. It has to be noted that even the relatively low doxycycline dose used in our experiments resulted in EGFP-HAS3-MV3 cells in relatively high secretion of hyaluronan comparable to cells such as mesenchymal cells (Qu et al., 2014). It is thus possible that the observed antiproliferative effect from HAS3 induction in our experiments is specific for this high hyaluronan level, and the induction with a lower stimulation of hyaluronan production could have either failed to inhibit proliferation or could even have stimulated it. However, our results are in line with studies from naked mole rats which express extremely HMW hyaluronan and are cancer free. High molecular weight hyaluronan in these rats provoke the activation of a special form of INK4a/b locus called pALTINK4a/b, which induces cell cycle arrest upon a variety of stresses (Tian et al., 2013, Tian et al., 2015). Similar results were shown in vascular smooth muscle cells where HMW hyaluronan inhibited cell cycle progression by repressing GTP loading of RAC and cyclin D1 activity (Kothapalli et al., 2008). However, these studies are in contrast to a report where exogenous hyaluronan induced cyclin D1 activity and proliferation in mesenchymal stem cells (Liu et al., 2016b). There is still some co-regulation in cyclins and hyaluronan synthesis, since inhibiting cyclin D3 also inhibited hyaluronan production in glucose stimulated mesangial cells (Ren, Hascall & Wang, 2009). Interestingly in our results, reduced proliferation was reversed by treating HAS3 overexpressing cells with *Streptomyces* hyaluronidase, which degrades the pericellular hyaluronan coat, indicating that the antiproliferative effect was due to increased hyaluronan production. *Streptomyces* hyaluronidase, by removing most of the cell surface hyaluronan coat, may modulate cell adhesion, for example by integrins (Chopra et al., 2012), and thereby affect migration and proliferation. In MCF7 cells exogenous hyaluronidase induced cell proliferation by activating MAPK-ERK and PI3K-AKT signaling (Hanoux et al., 2018) and accelerated migration and proliferation in fibroblasts during wound healing (Fronza et al., 2014) which is in line with our results. Using hyaluronan oligosaccharides (HA6), which compete with the binding of hyaluronan to CD44 (Knudson et al., 1996) but do not release hyaluronan from the synthase (Rilla et al., 2008), showed no significant difference in the proliferation of HAS3 overexpressing cells. This result indicates that the pericellular hyaluronan bound to its
synthase (HAS3), or to another hyaluronan receptor(s) other than CD44, was responsible for reduced cell proliferation. We showed that ERK1/2 phosphorylation was decreased in HAS3 overexpressing MV3 cells which has an effect in cell proliferation and growth control (Nishimoto, Nishida, 2006). This suggests that increased hyaluronan synthesis by HAS3 in melanoma cells reduces cell growth by inhibiting ERK1/2-signaling. This effect could relate to the large size of hyaluronan produced by the HAS3 overexpressing cells, which is also supported from HAS1–3 overexpression studies in vascular smooth muscle cells (Wilkinson et al., 2006). Similarly, in BRO melanoma cells, LMW hyaluronan of 30–50 kDa in size, but not HMW hyaluronan of 500–750 kDa in size, induce cell proliferation via CD44 (Sapudom et al., 2017). However, the effect of hyaluronan molecular size seems to be cell type specific, and dependent on the nature of it (exogenous versus endogenous), as HMW hyaluronan stimulated and LMW hyaluronan inhibited the proliferation of perimysial fibroblasts from extraocular muscles (Ma et al., 2018).

**6.2.2 Increased hyaluronan synthesis reduces melanoma cell migration and adhesion**

Cell motility is a crucial feature for the ability of melanoma cells to invade and migrate to the surrounding tissues. Our data showed that migration capacity in HAS3 overexpressing cells was reduced in a scratch wound assay, and even more obviously in random cell migration experiments. Reduced migration could also be explained by a lowered ability to form focal adhesions in EGFP-HAS3 cells, as our experiments revealed reduced adhesion of the cells to collagen matrices. For cancer cell extravasation, early adhesion is important in tethering of the endothelial wall. The study by Laurich et al. (2004) showed that tumor cells, which express a large pericellular hyaluronan coats, were more adhesive than cells where synthases were inhibited with antisense HAS2 or HAS3 or the pericellular hyaluronan coat was degraded (Laurich et al., 2004). A similar effect was seen with prostate adenocarcinoma cells. In these cells, adhesion to bone marrow endothelial cells was decreased if the pericellular hyaluronan coat formation was prevented (Simpson et al., 2002). However, in our model the removal of the pericellular hyaluronan with hyaluronidase treatment did not restore the cells adhesion capacity. This result suggests formation of stable adhesion complexes or signaling cascades aside from the direct effect of hyaluronan. Focal adhesions play an important function in cell movement, survival and even in transcriptional regulation (Kleinschmidt, Schlaepfer, 2017). Our data showed reduced number of focal adhesions at the protruding edges in HAS3 overexpressing cells. The mechanism for the reduced focal adhesions remains unknown, but it could involve altered signaling due to an increased pericellular hyaluronan or increased HAS3 at the plasma membrane, which could interfere with the formation of focal adhesions.

Treatment of HAS3 overexpressing melanoma cells with hyaluronan oligosaccharides (HA6) to block the hyaluronan-CD44 interaction (Knudson, 1993) slightly restored their migratory capacity. This suggests that the reduced migration was partly mediated by the hyaluronan-CD44 interaction. Similarly, degradation of the pericellular hyaluronan coat with *Streptomyces* hyaluronidase in HAS3 overexpressing cells totally restored migration capacity, indicating that the pericellular hyaluronan does indeed reduce cell migration. The stimulatory effect on cell migration by the hyaluronidase could alternatively indicate that formation of fragments, or LMW hyaluronan, changes the receptor from CD44 towards another receptor, such as RHAMM; this is similar to malignant pleural mesothelioma cells with LMW hyaluronan of 15–40 kDa (Shigeeda et al., 2017) or in human choriocarcinoma cells (Mascaro et al., 2017). It remains to be investigated whether similar receptor switching occurs in our model using degradation of the endogenous pericellular hyaluronan coat. The signaling mechanism how the HA6 or *Streptomyces* hyaluronidase restores the migration capacity also remains to be elucidated. Hyaluronan oligomers of 3–9 disaccharides bind CD44 monovalently and displace endogenous hyaluronan from the receptor. This can lead to reduction in constitutive signaling via other receptors modulated by CD44, such as growth factor receptors (Toole, 2009). Increased or decreased signaling is anticipated depending on the receptor involved. For example, the
influence of hyaluronan-CD44 interaction is inhibitory for PDGFR (Porsch et al., 2014, Li, Heldin & Heldin, 2006). Furthermore, as hyaluronan attached to both synthase and CD44 restricts the mobility of CD44 (Freeman et al., 2018), and probably modifies the signaling functions, release of CD44 from HAS-complex by HA6 is also expected to modify the signaling. Digestion of hyaluronan with *Streptomyces* hyaluronidase also releases CD44 from the hyaluronan, causing similar changes in signaling. Hyaluronan oligosaccharides may also signal through other receptors, such as TLRs which do not recognize macromolecular hyaluronan. Thus, in chondrocytes the activation of NF-κB and p38 signaling pathways by HA6 were only partially dependent on CD44, whereas in rheumatoid synovial fibroblasts’ activation was mediated by both CD44 and TLR-4 (Hanabayashi et al., 2016, Ohno et al., 2006).

6.2.3 Induced HAS3 overexpression changes MAPK-kinase signaling in melanoma cells

As live cell imaging showed HAS3 protein synthesis and the formation of the pericellular coat is fast, we were interested to screen the signaling cascades activated during this time with a phosphokinase array. The array result indicated that both Src kinase and STATs were decreased by the induction of EGFP-HAS3 and hence hyaluronan synthesis. Src kinase phosphorylation showed decrease only early on, whereas the phosphorylation of STAT3, -5a/b and -6 were decreased both at 2 and 6 h time points. Interestingly, in the latter time point, the phosphorylation of STAT1 and STAT4 were increased in HAS3 overexpressing cells compared to uninduced cells. Broad immunohistochemical analysis of STATs from tissue specimens of primary oral malignant melanoma samples showed intense staining for STAT3 (Nikitakis et al., 2018). It is suggested that STATs play a role in melanomagenesis, and especially STAT3, which was found to be activated via tyrosine kinases such as Src (Niu et al., 2002). This finding is in line with our data, where we detected decreased phosphorylation in both Src kinases and STATs. STAT1 is reported to balance STAT4 in IFN-mediated activation of natural killer cells (Miyagi et al., 2007) and it can also activate IL-12 production, which mediates adaptive immunity T-helper 1 cells differentiation (Thierfelder et al., 1996). STAT1 activity was also linked to the IFN immunotherapy response and STAT1 deficiency leads to IFN-resistance in melanoma (Amalraj et al., 2013). The inhibitory influence of HAS3 overexpression on Src and STAT activation raises an interesting question about the homeostatic nature of high molecular weight hyaluronan and increased endogenous hyaluronan synthesis in melanoma cells. It would be interesting to explore if manipulation of endogenous hyaluronan synthesis could make the melanoma cells more sensitive to immunotherapy, and to induce host immune attack and expose them to phagocytosis?

Array screening also pointed towards reduced phosphorylation of MAP kinases, such as p38 at 6 h. Similar results were obtained from reduced ERK1/2 phosphorylation using Western blot in HAS3 overexpressing cells. The reduced ERK1/2 signaling supports our proliferation data, where we saw reduced cell doubling in HAS3 overexpressing cells. ERK signaling has been connected to melanomagenesis. N-cadherin mediated ERK1/2 and PI3K/AKT activity contributed to invasive potential in melanoma cells (Ciolczyk-Wierzbicka, Laidler, 2018) whereas inhibition of p38 and ERK1/2 pathways suppresses inflammation-induced metastasis in melanoma (Tang et al., 2018). Furthermore, inhibition of ERK1/2 signaling in melanoma mouse models sensitized tumors to radiotherapy (Kalal et al., 2018). These studies point out that melanoma cells could be less aggressive, when the phosphorylation of ERK1/2 and p38 is reduced, and is in line with our findings connecting ERK inhibition to reduced proliferation. This raises new insights into the relevance of hyaluronan in melanomagenesis and how pericellular hyaluronan can signal downstream from the plasma membrane to either induce or weaken the inflammatory impact, depending on its molecular size. Further studies are still necessary, especially to explore how HAS3 overexpression can modulate the tumor microenvironment and possibly sensitize melanoma cells to immunity and to therapy.
6.3 HYALURONAN IN THE MELANOMA TUMOR STROMA

The level of hyaluronan in the tumor stroma is often increased, correlating with increased metastasis and poor patient survival in many types of cancers, including breast (Auvinen et al., 2014), epithelial ovarian cancer (Anttila et al., 2000) and thyroid carcinoma (Böhm et al., 2002). Fibroblasts in the tumor stroma are activated to so-called CAFs, which modify the tumor microenvironment suitable for the cancer cells, often by producing hyaluronan (Itono, Zhuo & Kimata, 2008, Kalluri, Zeisberg, 2006). CAFs are activated by the growth factors and cytokines secreted by the cancer cells and this crosstalk between stromal fibroblasts and cancer cells is essential for cell invasion and metastasis (Augsten, 2014). In line with these findings our experiments with conditioned medium (CM) collected from C8161 melanoma cells showed highly upregulated hyaluronan synthesis in dermal fibroblasts via increased \( \text{HAS2} \) transcription. Interestingly, both HMW and LMW hyaluronan were increased in the melanoma CM-treated fibroblasts.

### 6.3.1 PDGF-receptor activation leads to \( \text{HAS2} \) expression in dermal fibroblasts

Previously, PDGF-BB and TGF-β were demonstrated to highly stimulate hyaluronan synthesis in fibroblasts, whereas EGF and bFGF have lower stimulatory effects (Heldin, Laurent & Heldin, 1989). Similarly, bFGF, EGF, PDGF-BB and TGF-β1 increased \( \text{HAS2} \) expression and hyaluronan synthesis in skin fibroblasts whereas TGF-β1 enhanced \( \text{HAS1} \) expression (Nagaoka et al., 2015). PDGF-BB increases \( \text{HAS2} \) expression in dermal fibroblasts (Li et al., 2007), which melanoma cells are known to secrete (Godden, Edward & MacKie, 1999). Activation of PDGFRα by melanoma cells secreted PDGF’s also lead to increased \( \text{HAS2} \) transcription and hyaluronan secretion (Willenberg et al., 2012), which is also in line with our results. Our results show that melanoma cell CM-treatment activated PDGFRα and -β and EGFR to a lesser extent. Furthermore, we confirmed the dependence of \( \text{HAS2} \) activation and increased hyaluronan synthesis on PDGFR activation by using an inhibitor against the PDGFR or siRNA against \( \text{PDGFRα} \) and -β. PDGF-AA, -AB, -BB, -CC and -DD are ligands for PDGFRα and -β, and activation induces intracellular signaling cascades, such as phosphorylation of JNK and p38 (Heldin, Lennartsson & Westermark, 2018). In line with this, we also showed that CM-treatment activates p38 and AKT phosphorylation in fibroblasts. However, Nagaoka et al. showed in skin fibroblasts that TGF-β1 is the most active of different cytokines to stimulate \( \text{HAS1} \) and \( \text{HAS2} \) expression, signaling via MAPK-p38, ERK1/2 and SMAD3 pathways (Nagaoka et al., 2015). Of the signaling inhibitors tested in our model, the strongest suppression of hyaluronan secretion was obtained with the AKT and p38 inhibitors. The results from our experiments indicate that AKT and p38 are the main signaling cascades leading to \( \text{HAS2} \) upregulation via PDGFR activation in fibroblasts after melanoma CM-treatment.

### 6.3.2 Morphological changes in fibroblasts correlate with increased invasion

Treatment of fibroblasts with CM markedly changed their morphology towards more elongated cells with an increased number of plasma membrane protrusions. These changes in morphology suggested that CM-treated fibroblasts may be more motile; therefore we studied their invasion into a 3D collagen-Cultrex matrix. Our results indicated that the invasion of these cells was indeed elevated which was associated with increased expression of MMPs -1, -2, -9 and -14. We were able to block CM-induced invasion by silencing either the PDGFR with an inhibitor or with siRNA or silencing \( \text{HAS2} \). This was due to a reduction in MMP-9 and \( \text{HAS2} \) expression after PDGFR silencing, indicating an impact for MMP-9 and HAS2 in fibroblast invasion. There may also be other factors present in the CM that are needed to act co-operatively with PDGF. However, our preliminary data shows that PDGF-BB upregulates \( \text{HAS2} \) expression and induces cell locomotion (invasion, migration) and cell elongation. \( \text{HAS2} \) silencing inhibits these effects,
strongly supporting the conclusion that PDGF is the major factor in CM, inducing HAS2 expression, hyaluronan synthesis and cell motility.

The effect of hyaluronan in the formation of CAFs has been speculated. Zhang et al. (2016) showed CAFs extracted from oral squamous cell carcinomas express high levels of HAS2, which is associated with α-SMA-positivity and MMP-1 activity (Zhang et al., 2016). Activation of PDGFRα in stromal fibroblasts is associated with increased hyaluronan and collagen deposition in mammary fat pad fibrosis and leads to overall stiffness of the mammary gland. Cancer cells injected into this microenvironment formed larger tumors, indicating that PDGFRα signaling in stromal fibroblasts and increased hyaluronan in the surrounding, interfere with normal ECM synthesis and produce a suitable microenvironment for tumor development (Hammer et al., 2017). This result is in line with our data that melanoma cells secreted factors, especially PDGF-BB, via PDGFRα and -β activation increases HAS2 expression and hence hyaluronan synthesis in fibroblasts. Elevated HAS2 expression seems to be associated with fibroblast activation to highly motile and matrix remodeling type, which kind of cells are found in the tumor stroma. Hyaluronan in the ECM mediates stromal cell invasion, motility and ECM remodeling which accelerates the formation of a suitable microenvironment for tumor growth (Hammer et al., 2017, Zhang et al., 2016) or even drug resistance. As shown in 3D hydrogel models, hyaluronan-rich ECM was able to weaken the efficiency of BRAF and MEK inhibitors used in melanoma therapy (Blehm et al., 2015). Cancer-associated fibroblasts can also mask the tumor cells from immune cell-mediated killing (Ziani et al., 2017). Ultimately, it is the stromal cells and their phenotype that determine cancer progression. Stromal fibroblasts, still at the primary state are able to prevent tumor cell growth and their dissemination. When the stromal cells are transformed to CAFs due to microenvironmental factors from the tumor cells, these CAFs support the growth and spreading of the tumor cells (Zhou et al., 2016, Izar et al., 2016).

As shown in the work of Siiskonen et al. (2013), hyaluronan is abundant in the metastatic melanoma stroma, but not in the tumor tissue, which is probably due to decreased HAS1 and HAS2 and increased HYAL-2 expression in melanoma cells (Siiskonen et al., 2013). Similar to Willenberg et al. (2012) and our results, melanoma cells in vivo could secrete growth factors which stimulate and activate stromal fibroblasts to produce hyaluronan, thereby modulating the tumor microenvironment. Tumor-associated hyaluronan can be fragmented by hyaluronidases in the microenvironment producing hyaluronan fragments of different length, as shown in chronic inflammation (Avenoso et al., 2018, Soroosh et al., 2016) or in wound closure (Tolg, Telmer & Turley, 2014). These hyaluronan fragments in the tumor ECM may possibly activate melanoma cells as well, as shown by Voelcker et al. (2008). Hyaluronan content in the ECM on other hand can mediate stromal cells invasion, motility and ECM remodeling, thereby accelerating a suitable microenvironment for tumor growth (Hammer et al., 2017, Zhang et al., 2016). Using size exclusion chromatography, we showed that the amount of LMW hyaluronan was increased in CM-treated fibroblasts, which could also correlate with the in vivo tumor microenvironment.

Interestingly, our results also show that melanoma CM-treatment induced the formation of hyaluronan-positive cable-like structures in dermal fibroblasts. Hyaluronan cables have a proposed function in CD44-dependent leukocyte adhesion during inflammation, hyperglycemia and ER-stress (de La Motte et al., 1999, Jokela et al., 2008b), and they are also suggested to recruit macrophages and monocytes to the tumor area (Itano, Zhuo & Kimata, 2008). Leukocyte binding has been shown to thicken these hyaluronan cables and increase the accumulation of hyaluronan in the microenvironment (Lauer et al., 2013), which in turn modulates the microenvironment favorable for tumor cells as well as immune cells polarization towards immunosuppressive (Kuang et al., 2007, Itano, Zhuo & Kimata, 2008). The idea that melanoma cells secrete factors which induce the formation of monocyte binding hyaluronan cables, is interesting and our results partly support this. While melanoma cells do not produce much hyaluronan by themselves, they efficiently induce surrounding cells, such as fibroblasts,
to produce hyaluronan and thereby modify the microenvironment suitable for melanoma progression.
7 Summary and conclusions

The purpose of this doctoral thesis was to determine the relevance of hyaluronan in melanoma and its effect in the early stages of melanomagenesis.

The main findings of this thesis are:

- UVB decreases hyaluronan secretion from primary melanocytes in a dose-dependent manner, but does not influence the pericellular hyaluronan coat
- UVB and hyaluronan fragmentation with *Streptomyces* hyaluronidase induce IL-6, IL-8, CXCL-1 and CXCL-10 expression and secretion via TLR-4-NF-κB signaling in primary melanocytes
- HAS2 is the main enzyme producing the pericellular hyaluronan coat as well as the secreted hyaluronan in primary melanocytes
- Upon overexpression in melanoma cells, EGFP-HAS3 is rapidly produced and active at the plasma membrane
- HAS3 overexpression produces HMW hyaluronan in melanoma cells
- In metastatic melanoma cells, HAS3 overexpression reduces metastatic melanoma cell proliferation by reducing ERK1/2 signaling due to increased pericellular hyaluronan
- HAS3 overexpression reduces melanoma cell migration, partly via hyaluronan-CD44 interaction
- Melanoma cells secrete growth factors that activate dermal fibroblasts PDGFR leading to AKT and p38 induced HAS2 expression and increased hyaluronan synthesis
- Melanoma cell induced HAS2 expression and hyaluronan synthesis in dermal fibroblasts is associated with altered cell morphology and enhanced invasion into 3D-matrix

This thesis focused on studying the significance of hyaluronan in melanoma development and progression and provides novel findings. The results show that melanocytes secrete high amounts of hyaluronan and contain thick pericellular hyaluronan coats. UVB-exposure, especially when combined with the fragmentation of the pericellular coat by hyaluronidase addition, induces the expression and secretion of proinflammatory mediators in HAS2 and hyaluronan dependent manner. This may contribute to melanocytes transformation. During melanoma progression hyaluronan levels are declined. By increasing melanoma cells hyaluronan synthesis their proliferation and migration are reduced. In cancer stroma, factors secreted by the melanoma cells induce fibroblasts activation and hyaluronan synthesis. This microenvironmental hyaluronan released by the fibroblasts can act as a suitable milieu for melanoma cell spreading. These results help us to understand the significance of hyaluronan in the early and in more advanced stages of melanoma that could be used as potential target for melanoma therapy.

Moreover, the thesis provides new avenues for future research on how to detect melanoma development at early phases or how to prevent metastatic melanoma growth. In conclusion, hyaluronan has an evident role in melanomagenesis and further studies should concentrate on its impact in the early phases of melanoma.
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The thesis showed that UVB, the most important risk factor of skin melanoma, induces the expression of genes involved in melanomagenesis in hyaluronan-dependent way. While increased hyaluronan synthesis reduced growth of metastatic melanoma cells. Factors secreted by melanoma cells activated fibroblasts to produce hyaluronan that modulates the microenvironment suitable for tumor growth. The results help to understand the role of hyaluronan in melanomagenesis and its potential as a therapeutic target.