PUBLICATIONS OF
THE UNIVERSITY OF EASTERN FINLAND

UNIVERSITY OF
EASTERN FINLAND

Dissertations in Health Sciences

PIERRE R. MOREAU

EXPLORING THE NON-CODING RNA LANDSCAPE IN ATHEROSCLEROSIS: CELLULAR AND STIMULUS-SPECIFIC PROFILING OF KEY REGULATORY ELEMENTS

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To be presented by permission of the Faculty of Health Sciences,
University of Eastern Finland for public examination in MS300 Auditorium,
Kuopio on May 8th, 2024, at 9 o'clock

Publications of the University of Eastern Finland
Dissertations in Health Sciences
No 825

A.I. Virtanen Institute for Molecular Sciences
University of Eastern Finland, Kuopio
2024

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Printing office
PunaMusta Oy, 2024
Distributor: University of Eastern Finland
Kuopio Campus Library
www.uef.fi/kirjasto

ISBN: 978-952-61-5199-1 (print/nid.)
ISBN: 978-952-61-5200-4 (PDF)
ISSNL: 1798-5706
ISSN: 1798-5706
ISSN: 1798-5714 (PDF)

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Moreau, Pierre R.
Exploring the non-coding RNA landscape in atherosclerosis: cellular and stimulus-specific profiling of key regulatory elements
Kuopio: University of Eastern Finland
Publications of the University of Eastern Finland
Dissertations in Health Sciences 825. 2024, 175 p.
ISBN: 978-952-61-5199-1 (print)
ISSNL: 1798-5706
ISSN: 1798-5706
ISBN: 978-952-61-5200-4 (PDF)
ISSN: 1798-5714 (PDF)

ABSTRACT

Atherosclerosis is one of the leading causes of death in our society today. It starts with vascular endothelial cell (EC) dysfunction, induced by factors such as elevated blood cholesterol levels, hypertension, and smoking. EC dysfunction triggers the expression of inflammatory mediators which act to recruit monocytes which subsequently enter the tissue and differentiate into macrophages (Mϕs). As the disease progresses, Mϕs start to engulf oxidized lipids, transforming them further into foam cells. Finally, the migration of the smooth muscle cells (SMCs) leads to a thickening of the arterial wall, which subsequently produces a hypoxic microenvironment *in situ*. Further advanced atherosclerosis involves SMC apoptosis increasing the risk of plaque rupture, thrombosis and myocardial infarction. Overall, atherosclerosis is a chronic inflammatory disease resulting from intricate interactions between the different cell types within the vascular wall and immune cells.

Numerous genes have been shown to be implicated in atherosclerosis, yet understanding how these genes operate differently in ECs, Mϕs, and SMCs, during atherogenesis, remains elusive. This is largely due to a lack of knowledge of how transcriptional gene programmes differ between cell types and contribute to disease progression.

Recently, non-coding RNAs (ncRNAs) have emerged as important regulators of cardiovascular diseases through their ability to modulate target gene expression. This thesis aims to investigate the regulatory roles of miRNAs and lncRNAs in atherosclerosis-relevant cell types and stimuli. In the first study, we made use of a wide range of next-generation sequencing techniques to study the nascent and stable transcriptome profiles of ECs, SMCs and Mϕs under various pro-atherogenic stimuli. We show that the majority of mature miRNAs are shared between the different cell types and that only a small subset of miRNA species differs between them. In addition, we demonstrated that the top 10 miRNAs are responsible for over 80% of the total miRNA abundance. In line with this, the majority of nascent pri-miRNA variants were also expressed in all cell types, whilst 16% of the miRNAs exhibited a multi-variant expression profile, where transcription start site usage was highly cell-type specific. Additionally, we show that pro-atherogenic stimuli drive the transcriptional expression of pri-miRNA through similar mechanisms. Finally, we validated the contribution of miR-100-5p towards the inhibition of the HIPPO signaling pathway in ECs, leading to the activation of the YAP/TAZ cascade.

In the second study, we analyzed the transcriptional changes caused by hypoxia in ECs and investigated their long non-coding RNA profiles. We demonstrated that hypoxia regulates the nascent transcription of ~1800 lncRNAs and showed that promoter associated lncRNAs are more likely to be induced compared to enhancer associated lncRNAs. This difference could be attributed to differential activities of HIF1α and HIF2α and the binding of distinct transcription factors upon hypoxic stimulution. We also determined that hypoxia leads to a significant induction in the activity of superenhancers next to genes implicated in angiogenesis, cell survival and cell adhesion, alongside an enrichment of specific transcription factor binding motifs from genes involved in these processes. Conversely, superenhancers near regulators of peptidyl-tyrosine dephosphorylation, signal transduction and GTPase activity were repressed. Furthermore, we demonstrate that many of the lncRNAs identified *in vitro* were also differentially regulated *in vivo* in both primary and restenotic human atherosclerotic lesions and may serve as potential biomarkers for lesional hypoxic status in patients.

Depending on their cellular localization, lncRNAs can have different roles. In the nuclear compartment, lncRNAs are key components of several biological processes. They play a critical role in chromatin organization, transcriptional and post-transcriptional gene expression, as well as acting as structural scaffold components of nuclear domains. In the cytoplasmic compartment, in contrast, lncRNAs affect mRNA turnover and translation, regulate protein stability, and modulate different signalling pathways. Thus, in the third, study we profiled the transcriptome of different cellular compartments of ECs under hypoxic conditions and studied the distribution of lncRNAs between them. We identified nearly two times more lncRNAs in the nuclear compartment compared to the cytoplasm and demonstrated a compartment specific response to hypoxia, with only 23% of lncRNAs being regulated in both compartments. Furthermore, we pinpointed the compartment of origin of coding and non-coding transcripts deregulated upon hypoxia in ECs. Ultimately, we examined the genome-wide effect of the knock-down of six nuclear enriched lncRNAs, *SDCBP2-AS1*, *A2M-AS1*, *SLC2A1-AS1*, *SZT2-AS1*, *SNHG15* and *LUCAT1* which are differentially expressed under hypoxic conditions and found that whilst *SDCBP2-AS1*, *SNHG15*, *SZT2-AS1* and *A2M-AS1* are involved in the regulation of inflammation, *SDCBP2-AS1*, *A2M-AS1*, *SLC2A1-AS1*, *LUCAT1* and *SZT2-AS1* are related to the control of proliferation. Furthermore, *SDCBP2-AS1*, *SLC2A1-AS1* and *SZT2-AS1* appear to influence the glycolytic pathway. Moreover, validation of their role in regulating cell proliferation of ECs shows that while *SNHG15*, *A2M-AS1* and *SLC2A1-AS1* have anti-proliferative effects, *LUCAT1* promotes the opposite effect.

In conclusion, these studies highlight the importance of non-coding RNAs in cardiovascular disease and provide new potential targets for therapeutic intervention, as well as becoming potential health and disease status biomarkers. Further investigation into the regulatory roles of miRNAs and ncRNAs in atherosclerosis-associated cell types may provide new insights into the pathogenesis of atherosclerosis, as well aid the development of diagnostic and/or treatments for this disease.

Keywords: cardiovascular disease, atherosclerosis, angiogenesis, long non-coding RNAs, microRNAs, endothelial cells, smooth muscle cells, macrophages, hypoxia, oxidized lipids

Moreau, Pierre R.
Ei-koodaavien RNA-molekyylien muutokset ateroskleroosissa: Solu- ja ärsykekohtaiset säätelymekanismit
Kuopio: Itä-Suomen yliopisto
Publications of the University of Eastern Finland
Dissertations in Health Sciences 825. 2024, 175 s.
ISBN: 978-952-61-5199-1 (nid.)
ISSNL: 1798-5706
ISSN: 1798-5706
ISBN: 978-952-61-5200-4 (PDF)
ISSN: 1798-5714 (PDF)

TIIVISTELMÄ

Ateroskleroosi on yksi johtavista kuolinsyistä yhteiskunnassamme tänä päivänä. Se alkaa verisuonten endoteelisolujen toimintahäiriöillä, jonka taustalla olevia tekijöitä ovat muun muassa kohonnut veren kolesterolitaso ja verenpaine sekä tupakointi. Endoteelin toimintahäiriö laukaisee tulehdusvälittäjäaineiden ilmentymisen mikä houkuttelee paikalle monosyyttejä, jotka tunkeutuvat kudokseen ja erilaistuvat makrofageiksi. Taudin edetessä makrofagit alkavat niellä hapettuneita lipidejä, mikä johtaa niiden muuntumiseen vaahtosoluiksi. Lopuksi sileälihassolujen migraatio valtimon seinämän keskikerroksesta edistää leesion muuttumista yksinkertaisesta rasvajuosteesta komplisoituneeksi leesioksi, johtaen valtimon seinämän paksuuntumiseen ja hypoksisen mikroympäristön muodostumiseen. Pitkälle edenneessä ateroskleroosissa sileälihassolut kuolevat apoptoottisesti, mikä johtaa plakkia peittävän fibroottisen kuoren ohentumiseen lisäten täten riskiä plakin repeytymiseen, tromboosiin ja sydäninfarktiin. Kaiken kaikkiaan, ateroskleroosi on krooninen tulehdussairaus, joka johtuu monimutkaisesta vuorovaikutuksesta verisuonten seinämän eri solutyyppien ja immuunisolujen välillä.

Monien geenien on osoitettu olevan yhteydessä ateroskleroosiin, mutta ymmärrys siitä, miten näiden geenien toiminta eroaa endoteeli-, makrofagi-

ja sileälihassoluissa on vähäistä. Tämä johtuu suurelta osin tiedon puutteesta siitä, miten transkriptionaaliset geeniohjelmat eroavat solutyypeittäin ja miten ne vaikuttavat taudin etenemiseen.

Ei-koodaavien RNA-molekyylien (ncRNA) on osoitettu olevan tärkeitä säätelijöitä sydän- ja verisuonitaudeissa, sillä ne kykenevät muuntelemaan kohdegeeniensä ilmentymistä. Tämä väitöskirja pyrkii tutkimaan mikro RNA:ien (miRNA) ja pitkien ei-koodaavien RNA:iden (lncRNA) säätelyrooleja ateroskleroosin kannalta olennaisissa solutyypeissä ja olosuhteissa. Ensimmäisessä osatyössä, hyödynsimme laajasti erilaisia syväsekvensointimenetelmiä tutkiaksemme endoteeli-, makrofagi- ja sileälihassolujen geenien ilmenemisprofiileja geeninsäätelyn eri vaiheissa. Osoitimme, että suurin osa valmiista miRNA:ista on samanlaisia eri solutyypeissä. Havaitsimme, että kymmenen yleisintä miRNA:ta muodostaa yli 80 % solujen miRNA:ista. Myös suurin osa esiaste-pri-miRNA-varianteista ilmeni kaikissa solutyypeissä, kun taas 16 %:lla näistä miRNA:ista oli useita eri variantteja johtuen solutyyppispesifisestä transkription aloituspaikan käytöstä. Lisäksi osoitimme, että pro-aterogeeniset olosuhteet ajavat pri-miRNA:en ilmenemistä samanlaisten transkriptionaalisten mekanismien kautta. Lopuksi osoitimme miR-100-5p:n estävän HIPPO-signalointireitin toimintaa endoteelisoluissa, johtaen YAP/TAZ-välitteisen signaaliketjun aktivaatioon.

Toisessa osatyössä, analysoimme hypoksian aiheuttamia transkriptionaalisia muutoksia endoteelisoluissa keskittyen lncRNA profiilien muutoksiin. Havainnollistimme, että hypoksia säätelee noin 1800 pitkän ei-koodaavan RNA:n (lncRNA) tuotantoa ja osoitimme, että promoottorialueisiin liittyvät lncRNA:t indusoituvat todennäköisemmin kuin tehostaja-alueisiin liittyvät lncRNA:t. Tämä eroavaisuus voidaan liittää HIF1α:n ja HIF2α:n erilaiseen aktiivisuuteen sekä erilaisten transkriptiotekijöiden sitoutumiseen hypoksiastimulaatiossa. Osoitimme myös, että hypoksia johtaa supertehostaja-alueiden toiminnan merkittävään lisääntymiseen angiogeneesiä, solujen selviytymisetä ja adheesiota säätelevien geenin läheistyydessä, kun taas peptidyyli-tyrosiinidefosforylaation, signaalinvälityksen ja GTPaasin toiminnan säätelijöiden lähellä sijaitsevien supertehostaja-alueinden toiminta väheni.

Tutkimuksissamme selvisi myös, että usean solumalleissa tunnistetun lncRNA:n ilmeneminen oli linjassa ihmisen primaari- ja restenoosileesioissa havaittuihin muutoksiin, jotka siten voisivat toimia potentiaalisina biomarkkereina tulevaisuudessa.

LncRNA:illa on erilaisia rooleja riippuen niiden sijainnista solussa. Tumassa sijaitsevat lncRNA:t ovat avainasemassa useissa biologisissa prosesseissa. Niillä on kriittinen rooli kromatiinin organisaatiossa, transkriptionaalisessa ja post-transkriptionaalisessa geenien ilmentymisessä, ja ne toimivat rakenteellisina tukiosina tuman eri osissa. Solulimassa puolestaan lncRNA:t vaikuttavat lähetti-RNA:n translaatioon ja hajotukseen, säätelevät proteiinien stabiilisuutta ja muovaavat signalointireittejä. Kolmannessa osatyössä, selvitimme muutoksia tuman ja soluliman lncRNA tasoissa hypoksialle altistetuissa endoteelisoluissa. Tunnistimme lähes kaksi kertaa enemmän lncRNA:ita tumassa verrattuna solulimaan ja osoitimme, että vaste hypoksiaan on riippuvainen lokaatiosta, sillä vain 23 % lncRNA:sta säädeltiin sekä tumassa että solulimassa. Lisäksi osoitimme missä osastoissa sijaitsevat ne koodaavat ja ei-koodaavat transkriptit, joiden säätelyä hypoksiastimulus ei endoteelisoluissa muuttanut. Lopuksi tutkimme kuuden tumassa rikastuneen ja hypoksiassa eri lailla ekspressoituvan lncRNA:n, *SDCBP2-AS1, A2M-AS1, SLC2A1-AS1, SZT2-AS1, SNHG15* ja *LUCAT1,* hiljentämisen genominlaajuisia vaikutuksia. Havaitsimme, että *SDCBP2-AS1, SNHG15, SZT2-AS1* ja *A2M-AS1* liittyvät tulehduksen säätelyyn, kun taas *SDCBP2-AS1, A2M-AS1, SLC2A1-AS1, LUCAT1* ja *SZT2-AS1* säätelevät osaltaan solujen proliferaatiota. Lisäksi *SDCBP2-AS1, SLC2A1-AS1* ja *SZT2-AS1* vaikuttivat glykolyysireitin toimintaan. Validaatiokokeet osoittivat että *SNHG15, A2M-AS1* ja *SLC2A1-AS1* hidastivat endoteelisolujen kasvua kun taas *LUCAT1*:lla oli päinvastainen vaikutus.

Yhteenvetona nämä tutkimukset vahvistavat ei-koodaavien RNA:iden merkitystä sydän- ja verisuonitaudeissa, jotka voisivat toimia terapeuttisina lääkekohteina tai biomarkkereina. Lisätutkimukset miRNA:ien ja lncRNA:ien säätelyrooleista ateroskleroosin liittyvissä solutyypeissä voivat siten tuoda uusia näkökulmia ateroskleroosin patogeneesiin, sekä auttaa diagnostiikan ja/tai hoitojen kehittämisessä tulevaisuudessa.

Avainsanat: sydän- ja verisuonitaudit, ateroskleroosi, angiogeneesi, pitkät ei-koodaavat RNA:t, mikroRNA:t, endoteelisolut, sileälihassolut, makrofagit, hypoksia, hapettuneet lipidit

ACKNOWLEDGEMENTS

The work presented in this thesis was conducted in the group of Minna Kaikkonen-Määttä at the A.I. Virtanen Institute for Molecular Sciences, Faculty of Health Sciences at the University of Eastern Finland, Kuopio. This doctoral journey has been an enriching experience and I am deeply grateful to all those who have helped me and shaped this experience.

First and foremost, I wish to express my deepest gratitude to my main supervisor, Professor Minna Kaikkonen-Määttä. I will always be immensely grateful for the opportunity to work in your research group, for your unwavering trust, encouragement, as well as the immense support you've consistently provided throughout my PhD project. Your guidance and knowledge have been an invaluable reference point for my development as a scientist, and your inspiring leadership has motivated me to persevere through the challenging moments.

I also extend my deep gratitude to my secondary supervisor, Maria Bouvy-Liivrand, for your invaluable contributions to my research. When I began this journey, I had extremely limited experience in bioinformatics. Working with you has been a rich learning experience, and I greatly appreciate your availability, patience, guidance and support which has been instrumental in creating and shaping my bioinformatic knowledge.

I want to express my appreciation to the official reviewers of this thesis, Assistant Professor Anniina Vihervaara and Docent Daniela Mennerich, for dedicating their time and effort to review my work. Your valuable comments and suggestions to enhance the quality of my thesis are sincerely acknowledged. I would also like to thank Thomas Dunlop for your linguistic revisions and Anu Nyberg for translating my thesis abstract into Finnish.

In addition, I am immensely grateful to Professor Lars Maegdefessel for accepting to be my official opponent at the public defence.

I would like to express my sincere gratitude to all my co-authors for their significant contributions and expertise. Their insights and collaboration

were essential in the development of the published papers and manuscripts. Particularly, I would like to thank Suvi Kuosmanen for her limitless knowledge of microRNAs and her ability to see the bright side of uncooperative datasets. In addition, I would like to thank Tiit Örd for his invaluable insights. His indefatigable dedication to rigorous scientific research has been a constant source of inspiration throughout my PhD journey. It is a commitment I aspire to emulate in my own scientific career.

This work would not have been possible without the collaboration and peer support of brilliant scientists, both past and present, who comprise the CADGEN group. Their contributions were essential to this work.

My time in Finland has been enriched not just by professional pursuits, but also by the gift of lasting friendships. I have had the privilege to connect with remarkable people who have become treasured friends. Thank you, Anu, my office accomplice, whose kindness and dedication to everyone around her is truly inspiring. She's always there to offer a listening ear and a helping hand, no matter the situation. Special thanks to Jesse, the legendary barbecue chef, whose impeccable sense of style (from sweatpants to well-fitted suits) serves as a constant source of inspiration, and Nutti whose playful presence and furry cuddles brightened the day.

Huge thanks to Mari, the whirlwind of energy who dragged me on epic adventures including the infamous Ice Marathon. Her kindness and humour are as endless as her stamina. Big thanks also to Ben, the voice of reason and calm amidst the chaos. His wisdom, strength and support were invaluable. And to Freya and Saga (the four-legged adventurer!), for adding joy to every outing.

A heartfelt thanks to Tom, who became a cornerstone of my Finnish experience. His selfless generosity, brilliant mind, infectious humour, and booming laugh brightened even the gloomiest Finnish day.

Thank you, Eloi, the brilliant young scientist with a penchant for wild escapades. His humour and adventurous spirit made Finland even more memorable.

A big thanks to Nick, my unlikely British compatriot in Kuopio. We shared many laughs and good times together. His dry wit and sense of mirth made our time in Finland even more pleasant.

A special thanks to Maykel, his constant encouragement fuelled my determination during all those challenging times, and I am truly thankful for the positive energy and infectious smiles you brought.

Earnest thanks to Flavia, whose stimulating intellect and unwavering passion for science made her a cherished friend and a valuable colleague.

I would also like to express the warmest thanks for Aarthi, Henri, Isidore, Kadri, Oscar and Petri for countless exciting moments, from sharing delicious dinners to invigorating hikes, fishing excursions and everything in between.

My sincere gratitude also goes to Tuula Salonen for her invaluable technical assistance and Helena Pernu for her support in navigating the complexities of administrative tasks.

My immense gratitude goes to Vanesa, my partner, a brilliant scientist and an exceptional colleague. Our collaboration on research projects and in life has been instrumental in the making of this work. Her brilliance, dedication, and relentless pursuit of excellence have been a constant source of inspiration. She made the best days eve brighter and the toughest ones, if not enjoyable, at least bearable. Her love and unconditional support mean everything to me. Having her by my side, in the lab and in life, is a true privilege, and I wouldn't be here without her.

My deepest gratitude goes to Professors Otto Merten and Ville Friman. The mentorship and opportunity to intern in their labs were instrumental in launching my PhD journey. Their brilliant minds, dedication, and impressive achievements continue to inspire me.

I am particularly grateful to Lionel for introducing me to the PhD opportunity in Kuopio. His belief in my potential opened the door to this incredible Finnish adventure.

Words cannot express my gratitude to my dearest friends, Cyprien, Pierre-Louis, Thibault and William. Their enduring support, even across vast distances, has been a constant source of strength and joy throughout the years. Their presence is like a homecoming, instantly erasing any separation of time or space. These cherished friendships profoundly shaped me and immeasurably enriched my life.

I dedicate this work to my parents, Marie-Hélène and Robert. Their support and encouragement have been the foundation of every success I've achieved and have been instrumental in shaping who I am today. Their belief in me allowed me to pursue my career abroad, and their love provided a constant source of strength, even when miles separated us. I am incredibly fortunate to have them in my life. A special thought goes to my grandparents. I wish they could be here to celebrate this achievement with me. I know they would be incredibly proud.

Montrichard, March 2024

Pierre R. Moreau

This study was supported by the much-appreciated grants from the Instrumentarium foundation, the Finnish Cultural Foundation, Central and North Savo Regional Fund, the Maud Kuistila Memorial Foundation, the Finnish Foundation for Cardiovascular Research, the Ida Montin Foundation, the Aarne Koskelo Foundation, and the Antti and Tyyne Soininen Foundation, as well as the Doctoral Program in Molecular Medicine of the UEF.

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications:

I Pierre R. Moreau, Vanesa Tomas Bosch, Maria Bouvy-Liivrand, Kadri Õunap, Tiit Örd, Heidi H. Pulkkinen, Petri Pölönen, Merja Heinäniemi, Seppo Ylä-Herttuala, Johanna P. Laakkonen, Suvi Linna-Kuosmanen* and Minna U. Kaikkonen*. Profiling of Primary and Mature miRNA Expression in Atherosclerosis-Associated Cell Types. Arteriosclerosis, Thrombosis, and Vascular Biology 41:2149–2167, 2021.

II Moreau P.R., Örd T.*, Downes N.L.*, Niskanen H, Bouvy-Liivrand M., Aavik E., Ylä-Herttuala S.**, Kaikkonen M.U.** Transcriptional profiling of hypoxia-regulated non-coding RNAs in human primary endothelial cells. Frontiers in cardiovascular medicine. 2018 Nov 5;5:159.

III Pierre R. Moreau, Vanesa Tomas Bosch, Tiia Turunen*, Pia Latinen*, Mari-Anna Vananen*, Tiit Örd, Suvi Linna-Kuosmanen, Eloi Schmauch, Minna U. Kaikkonen. Profiling nuclear and cytoplasmic non-coding transcriptomes in endothelial cells under hypoxia. *Manuscript*.

* and ** denotes authors with equal contribution

CONTENTS

ABBREVIATIONS

ABCA1	ATP Binding Cassette Subfamily A Member 1
ABCG1	ATP Binding Cassette Subfamily G Member 1
AGO	Argonaute
ARE	Antioxidant Response Element
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
ASCVD	Atherosclerotic cardiovascular disease
ASO	Antisense oligonucleotide
ATAC-Seq	Assay for Transposase-Accessible Chromatin using sequencing
BRD4	Bromodomain-containing protein 4
CAGE	Cap Analysis of Gene Expression
CAD	Coronary artery disease
ChIP-Seq	Chromatin ImmunoPrecipitation Sequencing
CVD	cardiovascular disease
DEG	Differentially expressed gene
DGCR8	DiGeorge syndrome critical region 8
EC	Endothelial Cells
ECM	Extracellular matrix
eIFs	Eukaryotic initiation factors
EndoMT	Endothelial-mesenchymal transition
eRNAs	Enhancer RNAs
FDR	False discovery rate
GEO	Gene Expression Omnibus
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRO-Seq	Global run-on sequencing
GSEA	Gene Set Enrichment Analysis
GWAS	Genome-Wide Association Study
HAEC	Human aortic endothelial cell
HASMC	Human aortic smooth muscle cells
HDL	High-density lipoprotein

HIF	Hypoxia Inducible Factor
HRP	horseradish peroxidase
HUVEC	Human Umbilical Vein Endothelial Cell
Inr	Initiator element
IPA	Ingenuity Pathway Analysis
KLF	Krüppel-like factor
Kb	Kilobase
LDL	Low-density lipoprotein
lincRNAs	Long intergenic ncRNAs
lncRNA	long non-coding RNA
Mϕs	Macrophages
M-CSF	macrophage colony stimulating factor
MECP2	methyl-CpG binding protein 2
miRNA	microRNA
MI	myocardial infarction
MMPs	matrix metalloproteases
mRNA	messenger RNA
Mtor	mechanistic target of rapamycin
ncRNA	non-coding RNA
NELF	Negative Elongation Factor
NGS	Next-Generation Sequencing
NK	Natural Killer
NRF2	Nuclear factor erythroid 2-related factor 2
oxLDL	oxidized LDL
OxPAPC	oxidation products of 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine 1
PAPC	1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine 1
PCR	Polymerase chain reaction
PHD	prolyl hydroxylase domain-containing protein
PIC	Preinitiation complex
PRC	Polycomb Repressive Complex
Pre-mRNA	Precursor mRNA
pre-miRNA	Precursor miRNA
pri-miRNA	Primary microRNA
p-TEF-b	Positive transcription elongation factor
RISC	RNA-induced silencing complex
RNA-Seq	RNA Sequencing
ROS	Reactive oxygen species
RT-qPCR	Quantitative reverse transcription
SE	Super-enhancer

siRNA	small interfering RNA
SMC	Smooth muscle cells
SNPs	Single Nucleotide Polymorphisms
SWI/SNF	SWItch/Sucrose Non-Fermentable
TAD	Topologically associated domain
TBP	TATA-binding protein
TF	Transcription factor
TGS	Transcriptional gene silencing
TK	Thymidine kinase
TLR	Toll-like receptors
TNRC6	Trinucleotide repeat containing 6
tRNA	Transfer RNA
TSS	Transcription Start Site
TTS	Transcription Termination Site
TLR	Toll-like receptors
TAZ	transcriptional coactivator with PDZ-binding motif
TV	transcript variant
UTR	Untranslated Region
VEGF	Vascular Endothelial Growth Factor
VHL	von Hippel-Lindau
YAP	yes- associated protein 1

1 INTRODUCTION

Atherosclerosis is one of the leading causes of death in our modern society (Mozaffarian *et al.*, 2016; Benjamin *et al.*, 2017). Early events of atherogenesis start with endothelial cell (ECs) dysfunction which can be caused by factors such as elevated levels of cholesterol and triglycerides in the blood, high blood pressure as well as smoking. Dysfunction of the endothelium increases localized oxidative stress which causes the formation of oxidized lipids (formed from the increased free radical production acting on lipids) and the expression of inflammatory mediators on the surface of ECs which recruit monocytes, which enter the tissue and differentiate into macrophages (Mϕs) (Nabel and Braunwald, 2012; Gimbrone and García-Cardeña, 2016). During the progression of the disease, Mϕs start engulfing the oxidized lipids excessively, turning them into foam cells. Finally, the transition from a relatively simple fatty streak to more a complex lesion is characterized by the migration of smooth muscle cells (SMCs) from the medial layer of the arterial wall into the plaque. This leads to a thickening of the arterial wall and the subsequent setting up of a hypoxic microenvironment within the plaque (Wong *et al.*, 2017). During advanced atherosclerosis, SMC apoptosis can lead to a thinning and destabilization of the fibrous cap, resulting in plaque rupture, with dispersed fragments circulating around the vasculature to cause thrombosis and myocardial infarction. Atherosclerosis is thus an inflammation driven disease resulting from complex interactions between the two main cell types of the vascular wall and immune cells.

Despite intense efforts into determining the precise roles of ECs, Mϕs and SMCs in atherogenesis, we are far from understanding how the differences in the cellular responses contribute to the disease process. This is, in part, due to a lack of knowledge of how non-coding RNA transcriptional patterns differ between cell types and limited knowledge of non-coding RNAs that could contribute to disease progression.

Traditionally, the study of atherosclerosis mechanisms has predominantly concentrated on the roles of protein coding genes. This focus

was rooted in the initial understanding of the genome, where protein-coding sequences were deemed to be the primary drivers of biological functions and disease development. However, this perspective underwent a significant transformation following groundbreaking revelations by the Human Genome Project projects (Venter *et al.*, 2001), FANTOM (Functional annotation of mammalian genome) (Carninci *et al.*, 2005) or ENCODE (Encyclopaedia of DNA Elements) (Dunham *et al.*, 2012). These transcriptomic studies have shown that cumulatively, 90% of the human genome is transcribed across different cell types and under a variety of conditions (Dunham *et al.*, 2012a; ENCODE Project Consortium *et al.*, 2020). However, only 2% of the genome encodes for genes that produce proteins whilst the majority is only transcribed. These non-coding RNAs can be divided into two main categories based on their length. Long non-coding RNAs include, but not limited to, promoter- and enhancer-associated lncRNAs (Hon *et al.*, 2017; Wu, Yang and Chen, 2017) while small RNAs are exemplified by the class known as microRNAs.

Particularly, long ncRNAs (lncRNA) and micro RNAs (miRNAs) have emerged as key regulatory molecules, exhibiting a high degree of cell type specificity, thereby offering a more nuanced understanding of cellular functions. Since their discovery, both lncRNAs and miRNAs have been shown to affect several cellular functions inherent to numerous diseases, such as cell proliferation, differentiation, and response to stress. Despite their abundance and critical regulatory roles, ncRNAs have not been extensively studied in the context of atherosclerosis. This gap in research highlights a pivotal area of potential, where exploring the functions of ncRNAs in various cell types could unravel new dimensions of atherosclerotic mechanisms and pave the way for innovative therapeutic strategies. This shift in focus from coding genes to the expansive world of ncRNAs marks a new era in our understanding of atherosclerosis, beckoning a more comprehensive exploration of our genome's uncharted territories.

This thesis work aimed to characterizing the non-coding RNA profiles and the gene regulatory processes that take place in ECs, Mϕs and SMCs in response to proatherogenic stimuli. We were able to establish that miRNA-related regulatory mechanisms contribute to atherosclerosis and identify

the processes by which proatherogenic-stimuli-responsive miRNAs affect atherogenesis in a cell-type-specific manner. Furthermore, this work provides new understanding as to how non-coding RNA contributes to the regulation of the transcriptome upon pro-atherogenic stimuli. Finally, we successfully determined the subcellular localization preferences of non-coding RNAs in response to stimuli and identified previously uncharacterized ncRNAs that influence cell proliferation. Collectively, these discoveries highlight the role of ncRNAs as drivers of disease progression.

2 REVIEW OF THE LITERATURE

2.1 ATHEROSCLEROTIC CARDIOVASCULAR DISEASE

Atherosclerotic cardiovascular diseases (ASCVD) are a leading cause of death worldwide. Cardiovascular diseases are defined as disorders affecting the heart and the blood vessels. The most common ASCVDs are coronary heart disease, cerebrovascular disease and peripheral heart disease, which represent diseases of blood vessels affecting the heart, the brain and the peripheral limbs (arms, legs and internal organs), respectively. Aside from asymptomatic manifestations, the first sign of clinically significant atherosclerosis often presents itself as a severe clinical event, such as stroke or myocardial infarction (MI).

2.2 ATHEROSCLEROSIS-LINKED CELLULAR PHENOTYPES

Atherosclerosis starts with endothelial dysfunction **(Figure 1)**. The endothelium is a barrier constituted of a monolayer of endothelial cells (ECs) lining arterial, venous, and lymphatic vessels. ECs are exposed to the movement of blood and are, thus, sensitive to hemodynamic flow. The disturbance of the blood flow from the laminar flow creates shear stress. This stress is believed to be the first change that leads to the formation of an atherosclerotic lesion (Stary, 2000; Virmani *et al.*, 2000; Gimbrone and García-Cardeña, 2016). Several risk factors can trigger or enhance endothelial dysfunction. These include elevated levels of cholesterol and triglycerides in the blood, high blood pressure and smoking. Consequently, ECs themselves become more permeable to lipids circulating in the blood (Gimbrone and García-Cardeña, 2016). Later, the oxidation of those lipids by reactive oxygen species generated from oxidative stress, inside the intima, (Panieri and Santoro, 2015) will lead to the expression of inflammatory mediators on the surface of ECs. Subsequently, monocytes, responding to the presence of these pro-inflammatory signalling molecules will enter the tissue and differentiate into macrophages (Mϕs). In an attempt to remove

the oxidized lipids from the lesion site, Mϕs will engulf and degrade them. However, if the oxidized lipids levels become too abundant, Mϕs are unable to degrade all of the lipids, which build up within the cells, turning the Mϕs into foam cells. The accumulation of foam cells creates fatty streaks **(Figure 1)**. In that regard, defective efferocytosis, the process of removing dead cells, is considered one of the key elements of atherosclerosis progression towards plaque rupture (Geng & Libby, 1995; Otsuka *et al.*, 2015; Schrijvers *et al.*, 2007; Schrijvers *et al.*, 2005; Tabas, 2010; Van Vré *et al.*, 2012). In human coronary plaques, the abundance of uncleared dead cells correlates with necrosis and inflammation (Geng and Libby, 1995; Schrijvers *et al.*, 2005; Tabas, 2010; Otsuka *et al.*, 2015).

The transition from fatty streaks to more complex lesions is characterized by the migration and proliferation of SMCs from the medial layer to the intima of the vessel wall **(Figure 1)**. Due to the increased number of cells in the intima of the vessel, the vessel wall becomes thicker resulting in hypoxia due to the poor diffusion of oxygen. Moreover, as the lesion progresses, SMCs, exposed to stimuli from Mϕs and activated ECs, synthesise extracellular matrix components within the intimal layer of the vessel wall generating a fibromuscular plaque. Ultimately, the advanced lesion is constituted of oxidized lipoproteins and cholesterol crystals shielded by a fibrous cap, alongside the mentioned cell types, as well as the left overs of dead cells (Gimbrone and García-Cardeña, 2016) **(Figure 1)**. During advanced atherosclerosis, SMC apoptosis can lead to a thinning of the fibrous cap, resulting in plaque rupture, dispersal of plaque fragments through the circulation which may lead to thrombosis and myocardial infarction at other sites, distant from the original plaque location.

The vascular wall is composed of three layers: the intima, the media and the adventitia containing various cell types. In addition to ECs, vascular SMCs, monocytes and the CD14+/ Mϕs derived from monocytes, pericytes, fibroblasts and other immune cells, such as, dendritic cells, T cells, B cells and natural killer (NK) cells have been shown to play a role in atherosclerosis (Burger *et al.*, 2022; Cochain *et al.*, 2018; Depuydt *et al.*, 2020; Gu *et al.*, 2019; Kim *et al.*, 2018; Winkels *et al.*, 2018). Furthermore, recent advances in single-cell sequencing have played a critical role in expanding our knowledge of the

cell type composition of various human tissues. Particularly, this has allowed the discovery of multiple subpopulations of cells associated with healthy and pathogenic conditions, including atherosclerosis (Depuydt *et al.*, 2020; Örd *et al.*, 2021). In this thesis, we will focus on understanding the transcriptional responses of ECs, SMCs and CD14+/ Mϕs to proatherogenic stimuli.

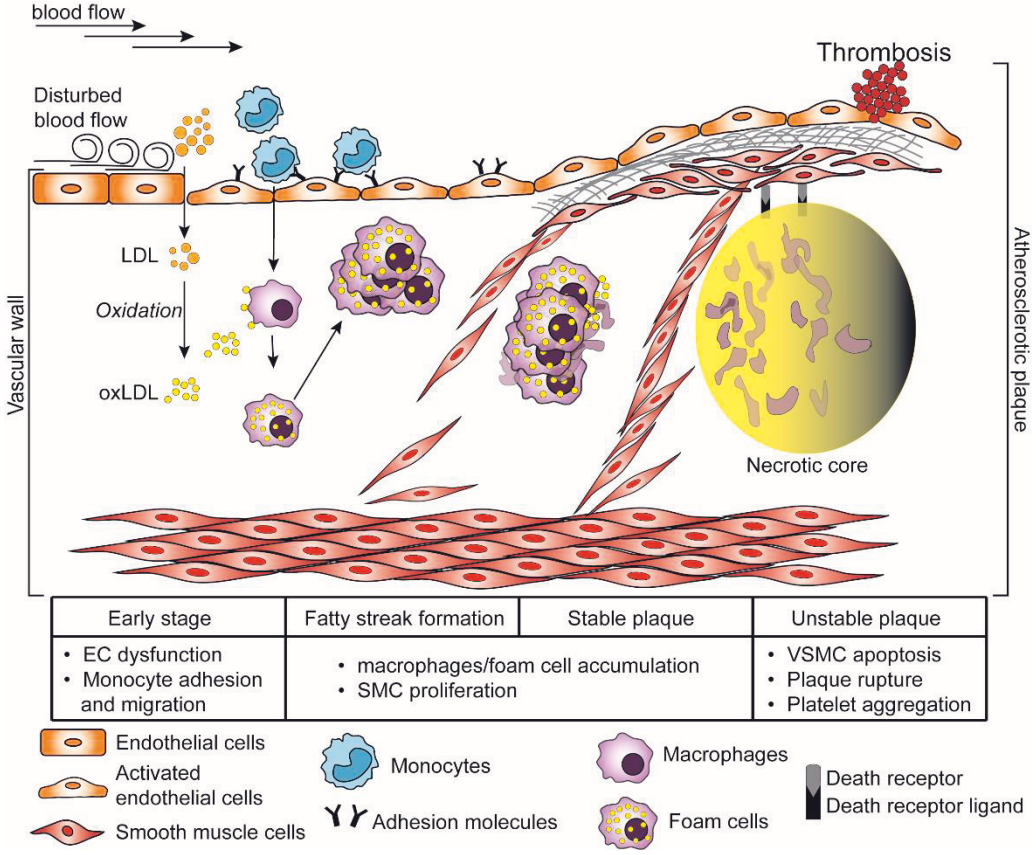

Figure 1. **Formation and development of an atherosclerotic plaque.** The process begins with injury to the endothelium, the innermost lining of the artery. This damage allows low-density lipoprotein (LDL) particles to seep into the subendothelial space. Here, LDL undergoes oxidation by hypoxia induced free-radical molecules, transforming it into oxidized LDL (oxLDL). Macrophages, derived from monocytes that infiltrate the damaged area, engulf oxLDL, becoming foam cells and initiating the formation of a fatty streak. Over time, the fatty streak evolves into a more complex fibrous plaque. This plaque features a necrotic core, composed of dead and dying cells, capped by a fibrous layer containing migrated vascular smooth muscle cells (VSMCs). Plaque rupture due to degradation of the fibrous cap can trigger thrombus formation and vessel blockage, potentially leading to

serious cardiovascular events. For clarity, only cell types used in the thesis are shown.

2.2.1 Endothelial cells (ECs)

As the main component of the cellular lining of all blood vessels, ECs form the interface between the systemic circulation and adjacent tissues. The main roles of this endothelium are to deliver oxygen and nutrients to surrounding tissues, regulate vascular tone and permeability, maintain haemostasis, prevent coagulation, control angiogenesis and regulate the inflammatory response (Cahill and Redmond, 2016; Ricard *et al.*, 2021). These functions of ECs are strengthened and modulated with the crosstalk with other vascular cell types such as SMCs and monocytes/Mϕs, and disturbances to these interactions are associated with vascular dysfunction and pathological remodelling in the progenitor phases of many cardiovascular diseases, such as atherosclerosis (Jaipersad *et al.*, 2014; Méndez-Barbero, Gutiérrez-Muñoz and Blanco-Colio, 2021).

ECs are exposed to blood flow and respond rapidly to haemodynamic flow disturbances via the action of mechano-sensor receptors that couple with intracellular signalling cascades and/or structural proteins (Baratchi *et al.*, 2017; Kwak *et al.*, 2014; Niu *et al.*, 2019). Prominent mechano-sensing proteins include PECAM-1 (CD31), Vascular endothelial (VE)-cadherin, VEGFR2 and VEGFR3 which trigger intracellular signalling pathways such as the PI(3)K and NFκB pathways to promote endothelial dysfunction (Tzima *et al.*, 2005; Conway *et al.*, 2013; Coon *et al.*, 2015). Moreover, perturbed blood flow results in an increased lipoprotein infiltration of the vessel intima due to the physical disruption of endothelial integrity and longer period contact with circulating LDLs (Kang, Cancel and Tarbell, 2014; Chistiakov, Orekhov and Bobryshev, 2017). Endothelial dysfunction can be amplified by exposure to bacterial or viral infection, hypoxic conditions, oxidized LDL, tobacco smoking and hyperglycaemia. All of these conditions prompt activation of ECs and the production of inflammatory factors such as interleukins, chemokines, colony stimulating factors, interferons, MCP-1 (monocyte chemoattractant protein-1), ICAM-1 (intercellular adhesion molecule-1), VCAM-1 (vascular adhesion molecule-1), and other proteins and growth

factors that will attract monocytes, which will subsequently enter the arterial wall (Abraham & Distler, 2007; Chistiakov *et al.*, 2018; Steyers & Miller, 2014). In that regard, reduction of inflammation has appeared as a therapeutic strategy to treat endothelial dysfunction, and consequently constrain cardiovascular disease development. For instance, activation of the anti-atherogenic transcription factors KLF2 (Krüppel-like factor 2) and KLF4 (Krüppel-like factor 4) increase eNOS (endothelial nitric oxide synthase) activity. This leads to the repression of NFκB transcription factor mediated transcription and activation (SenBanerjee *et al.*, 2004) as well as the repression of endothelial cell metabolism via repression of PFKFB3 (6-phosphofructo2-kinase/fructose-2,6-biphosphatase-3). This provokes a reduction in cellular glucose uptake, resulting in a metabolically quiescent state (Doddaballapur *et al.*, 2015; Eelen *et al.*, 2018) that could decrease or reverse endothelial dysfunction. Of note, KLF2 is also involved in the secretion of extracellular phospholipid vesicles containing mir-143/145, leading to the differentiation of vascular SMCs towards an anti-atherogenic phenotype (Hergenreider *et al.*, 2012).

Furthermore, a targeted approach involving the direct inhibition of key factors holds substantial promise in mitigating OxLDL-induced inflammation. For instance, the inhibition of TLR4 activation, a key inducer of the NFκB pathway, and subsequent suppression of NFκB itself, have demonstrated effectiveness in curtailing the secretion of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α (Wang *et al.*, 2016; Wang *et al.*, 2016; Xu *et al.*, 2016; Zhou *et al.*, 2020). Similarly, YAP (yes- associated protein 1) nuclear translocation, recognized for its association with the TEAD 1–4 transcription factor family and other binding partners, is involved in controlling the expression of genes implicated in cell cycle regulation and mitosis (Totaro, Panciera and Piccolo, 2018). Additionally, YAP is another target of interest, in order to reduce oxidized lipid mediated inflammation, as silencing of the HIPPO signalling pathway leads to dephosphorylation and subsequent activation of YAP and TAZ (transcriptional coactivator with PDZ-binding motif, also called WWTR1). The activation of these latter two TFs further propels cells toward pro-proliferative and pro-inflammatory responses, fostering conditions conducive to atherogenesis.

2.2.2 Macrophages (Mϕs)

In atherosclerotic lesions, plaque Mϕs largely originate from monocytes entering the intima of the vessel wall due to endothelial cell activation. Once in the subendothelial space, monocytes differentiate into Mϕs, following exposure to pro-differentiation factors such as M-CSF (macrophage colony stimulating factor) produced by activated ECs. When there, the Mϕs participate in the scavenging and uptake of oxidized lipoprotein particles and this turns them into foam cells (Smith *et al.*, 1995; Williams and Tabas, 1995). Indeed, elevated leucocytes and particularly monocytes have been associated with atherosclerosis progression and impaired lesion regression (Swirski *et al.*, 2007; Tacke *et al.*, 2007; Waterhouse, 2008; Nagareddy *et al.*, 2013). Studies have demonstrated that monocyte infiltration is increased throughout the progression of the disease (Swirski *et al.*, 2006) and that it is required for atherogenesis (Boring *et al.*, 1998; Gosling *et al.*, 1999), emphasizing the importance of monocyte-derived Mϕs in this process. Parenthetically, it is worth mentioning that populations of resident Mϕs are present in a number of organs, including the intima of the arterial wall. However, even though cardiac-resident Mϕs, for example, are self-maintained, upon atherogenesis, they are replaced by an influx of monocytes that will mature into Mϕs (Heidt *et al.*, 2014; Hilgendorf *et al.*, 2014; Perdiguero and Geissmann, 2016).

The classical view of Mϕs was to divide them into two main subtypes called M1 and M2. Phenotypically, M1 Mϕs are considered to be proinflammatory and are able to eliminate pathogens and cells infected by viruses or that have become transformed, whilst M2 Mϕs are anti-inflammatory and are essential in responses to parasites, tissue remodelling, angiogenesis, and allergic diseases (Martinez, 2008; Jenkins *et al.*, 2011). In this model, the M1 Mϕs are induced by IFN-γ (interferon γ), secretions of TH1 cells, cytotoxic T cells and NK cells, LPS (lipopolysaccharide), a component of the outer membrane of Gram-negative bacteria and GM-CSF (granulocyte-macrophage colony-stimulating factor) which stimulate the production of pro-inflammatory cytokines (Guha and Mackman, 2001; Fleetwood *et al.*, 2007; Billiau and Matthys, 2009). M1 Mϕs

pro-inflammatory properties rely on the secretion of cytokines such as TNFα, IL-1β, IL-12, IL-23, IL-6, chemokines, such as CCL-2 (CC- chemokine ligand 2) as well as Nitrous Oxide (NO, produced via iNOS) and reactive oxygen species (ROS), amongst others (Shapouri-Moghaddam *et al.*, 2018; Hoeksema and Glass, 2019). Conversely, the M2 phenotype is activated by stimuli such as, IL-4 and IL-13 (from Th2 cells), IL-10, TGF-β and CSF-1. The M2-Mϕs angiogenic and pro-fibrotic roles are acquired though the production of anti-inflammatory cytokines such as IL-10 and TGFβ (Shapouri-Moghaddam *et al.*, 2018; Hoeksema and Glass, 2019).

However, several recent studies have revealed that Mϕs phenotypes are not limited to these two phenotypes, but rather should be represented as a much broader, and continuous, spectrum involving differential cytokine production and functional characteristics induced by local microenvironments (Colin, Chinetti-Gbaguidi and Staels, 2014; Murray *et al.*, 2014; Xue *et al.*, 2014; Chávez-Galán *et al.*, 2015; Nahrendorf and Swirski, 2016). The development and application of single-cell sequencing techniques have allowed the corroboration of these findings, providing further evidence of the existence of a multitude of diverse phenotypes for Mϕs in tissues where they reside (Cochain *et al.*, 2018; Winkels *et al.*, 2018).

Amongst this spectrum of Mϕs, inflammatory subtypes represent the majority of the macrophage population within the intima of the atherosclerotic plaque and are considered to be the main drivers of inflammation in the lesion as well as being positively associated with plaque progression (Cole *et al.*, 2018; Kim *et al.*, 2018; Lin *et al.*, 2019; Willemsen & Winther, 2020). These inflammatory Mϕs generally express inflammatory marker genes, such as *Tnf*, *Nlrp3*, *Il1b*, *Egr1*, *Zpf36*, *Ier3*, *Cebpb*, *Cxcl2* and *Ccl2-5* in mouse and propagate TH1 and TH17 cell responses (Benoit *et al.*, 2008; Cochain *et al.*, 2018; Lin *et al.*, 2019; McArdle *et al.*, 2019). The inflammatory response in Mϕs is mediated by signal-dependent transcription factors such as NF-κB and the interferon regulatory factors (IRFs) such as IRF1, IRF5 and IRF8 (Günthner and Anders, 2013; Spiller *et al.*, 2016). Upon stimulation, transcription factors of the NF-κB family translocate to the nucleus and bind to NF-κB response elements on the DNA at accessible chromatin sites that have been previously set up by the lineage-determining transcription factors

PU.1 and C/EBPβ (CCAAT/enhancer-binding protein-β), whose action leads to histone acetylation, following the recruitment of histone acetyltransferase EP300, thereby permitting transcriptional activation (Ghisletti *et al.*, 2010; Liu *et al.*, 2017).

At the opposite end of the spectrum, Mϕs expressing TREM2 (Triggering receptor expressed on myeloid cells 2), a myeloid-specific transmembrane glycoprotein which interacts with apolipoproteins E and J, anionic ligands, glycerophospholipids and sphingomyelins that overall work to attenuate macrophage activation, has been shown to prevent infections, adipocyte hypertrophy, systemic hypercholesterolemia and body fat accumulation in mice as well as to be anti-correlated with human plaque stability (Turnbull *et al.*, 2006; Cannon, O'Driscoll and Litman, 2012; Rai *et al.*, 2016; Jaitin *et al.*, 2019). Mϕs expressing TREM2, called TREM2^{hi} Mϕs, were detected exclusively in atherosclerotic plaques during both the progression, in early and advanced lesions, as well as atherosclerotic lesion regression (Cochain *et al.*, 2018; Lin *et al.*, 2019). TREM2^{hi} Mϕs also expressed increased expression levels of *Spp1* (secreted phosphoprotein 1), *Cd9* (CD9 Molecule), *Cd36* (cluster of differentiation 36) and *Lgals3* (Galectin 3) amongst others, in the mouse (Cochain *et al.*, 2018; Kim *et al.*, 2018; Lin *et al.*, 2019). Pathway analysis of TREM2^{hi} Mϕs gene expression showed enrichment of oxidative phosphorylation and catabolism, lipid metabolism, regulation of cholesterol efflux, efferocytosis and tissue repair, suggesting an association with intracellular lipid accumulation as well as the consequential foamy lipid-laden macrophage formation (Cochain *et al.*, 2018; Kim *et al.*, 2018; Lin *et al.*, 2019).

Foam cells display a specific transcriptome profile with an increased expression of genes responsible for lipid metabolism through the activation of the transcription factors LXR, PPARγ (liver X receptor and peroxisome proliferator activated receptor γ, respectively), as well as their heterodimerisation partner RXR (retinoid X receptor), all of which are members of the steroid hormone nuclear receptor superfamily of transcription factors (Kim *et al.*, 2018). Activation of these transcription factors has been seen to partially inhibit the inflammatory response caused by the lipid activation of TLRs (Toll-like receptors) (Ogawa *et al.*, 2005). It

should be noted that LXR and PPARγ trans-repress distinct but overlapping subsets of proinflammatory genes (Ghisletti *et al.*, 2007). In addition, other transcription factors have been identified as essential for foam-cell formation and maintenance such as the AP1 class factor ATF3 which inhibits foam-cell formation by repressing the transcription of the *Ch25h* gene (encoding the enzyme cholesterol 25-hydroxylase) which leads to lower 25-hydrocholesterol formation and thus limit the levels of intracellular lipid levels (Gold *et al.*, 2012).

In developing atherosclerotic lesions, Mϕs actively participate in the uptake of lipoproteins particles with the expression of scavenger receptors that include SR-A (scavenger receptor type A), LRP1 (LDL receptor-related protein 1) and LOX1 (lectin-like OxLDL receptor 1) (Libby, 2000; Crucet *et al.*, 2013; Lillis *et al.*, 2015). In addition, Mϕs have the ability to engulf lipoproteins independently of receptors using a process called pinocytosis (Kruth *et al.*, 2005). The accumulation of foam cells advance both plaque growth and lipid build-up (Moore and Tabas, 2011; Moore *et al.*, 2018). Plaque Mϕs have been seen to engage in crosstalk with vascular SMCs, amplifying the inflammatory signal and instigating SMC migration, proliferation and phenotype-switching (Ross *et al.*, 1990; Campbell *et al.*, 1992; Cherepanova *et al.*, 2016; Li *et al.*, 2021). Furthermore, plaque Mϕs display lower migratory ability, increasing inflammation and further complicating the lesion. In addition, the combination of macrophage and foam cell apoptosis with defective efferocytosis leads to the accumulation of cell debris causing a necrotic core to form within the plaque (Moore, Sheedy and Fisher, 2013; Moore *et al.*, 2018; Bäck *et al.*, 2019).

2.2.3 Smooth muscle cells (SMCs)

In the vasculature, SMCs are located in the medial layer of vessel walls where they provide structural integrity. The SMCs are responsible for vessel contraction and dilation, blood pressure regulation, blood flow distribution as well as the production of extracellular matrix (ECM) components. In the aorta, SMCs are exposed to higher blood pressure, shear stress, and pulsatile blood flow, requiring a higher stiffness level (Yamin and Morgan,

2012). The contractile capacity of human aortic smooth muscle cells (HASMCs) is controlled by various substances originating from ECs, the autonomic nervous system, adrenal hormones, and reactive oxygen species.

Under normal conditions, SMCs express several contractile markers such as ACTA2 (α-actinin-2, also called α-SMA), TGLN (transgelin, sometimes referred as SM22α), MYH11 (cell myosin heavy chain 11, also known as SMMHC), and smoothelin (smooth muscle cell differentiation specific antigen otherwise known as SMTN) (Bennett, Sinha and Owens, 2016). However, SMCs have been seen to be able to modify their phenotype in response to structural or chemical damage of blood vessels. In such cases, SMCs are able to reduce contractility, whilst increasing their proliferative ability and the secretion of extracellular matrix components. This modified state of SMCs is also called the synthetic phenotype. The transformation process between these two states is bidirectional in nature, since after the injury is resolved and homeostasis is restored, SMCs are able to regain their contractile phenotype (Cao *et al.*, 2022).

During early atherosclerosis, after the formation of extracellular lipid pools in the intima of the vessel wall, HASMCs increase their secretion of ECM components, including primarily negatively charged proteoglycans (Ang *et al.*, 1990; Virmani *et al.*, 2000; Little *et al.*, 2002; Yahagi *et al.*, 2016; Langley *et al.*, 2017). The interaction between ECM components and the positively charged apolipoproteins induces the retention of the latter within the intima of the vessel wall (Skålén *et al.*, 2002; Thompson *et al.*, 2014), allowing the incorporation of apolipoproteins into LDL. If the lipid components of LDL are oxidised, monocytes are recruited to the plaque though increased secretion of chemokines, such as CC-chemokine ligand 2 and 5 (CCL2, CCL5) and CXC-chemokine ligand 1 (CXCL1) by ECs. The monocytes, in turn, differentiate into Mϕs (Napoli *et al.*, 1997).

Upon stimulation with inflammatory signals, intracellular lipid accumulation and other stimulators, SMCs undergo phenotype switching where they reduce expression of common SMCs markers and acquire Mϕs like markers such as CD68 and LGALS3, alongside an increased capacity for cell proliferation, migration and the secretion of various extracellular matrix proteins and cytokines.

Recent lineage tracing and single-cell transcriptome studies in atherosclerotic lesions have found that SMCs expressing LGALS3 were first transformed into fibroblast-like cells, called fibromyocytes (Wirka *et al.*, 2019). Fibromyocytes display a stem-cell and extracellular matrix-remodelling like phenotype, thereby, participating in the formation of the fibrous cap. Fibromyocytes are considered pioneer cells as they are able to differentiate into three other SMCs phenotypes, also called osteogenic phenotypes that may contribute to plaque calcification and plaque instability (Alencar *et al.*, 2020). Other studies have suggested that SMCs could transition into an intermediate pluripotent state called SEM (Stem cell + Endothelial cell + Monocyte) that could differentiate into macrophage-like and fibro-chondrocyte cells, or even restore their contractile phenotype (Pan *et al.*, 2020).

In the lipid-rich regions of human atherosclerotic plaques, several cells co-expressing CD68 and α-SMA, also have increased expression of ABCA1 (ATP-binding cassette transporter 1) and ABCG1 (ATP-binding cassette G1) which regulate cholesterol efflux, have been identified (Vengrenyuk *et al.*, 2015). Recent studies have shown that these cells originate from SMCs quiescent in the tunica media of the vessel wall that migrate to the intima and later transform into macrophage-like-SMCs (Bennett, Sinha and Owens, 2016). These SMCs, in a similar fashion to Mϕs, have the capacity to engulf lipids and necrotic cell debris, causing the formation of foam cells. However, due to lower expression of ABCA1, SMC-derived foam cells are unable to release cholesterol, and thus, are more likely to turn into foam cells compared to Mϕs, aggravating the formation of atherosclerotic plaques (Allahverdian *et al.*, 2014; Bennett, Sinha and Owens, 2016; Wang *et al.*, 2019). Moreover, SMC-derived foam cells have been shown to secrete MMPs (matrix metalloproteases), whose action causes the degradation of the extracellular matrix which leads to the weakening of the fibrous cap and the promotion of SMCs migration that eventually ends with pathological remodelling and vascular restenosis (Cho & Reidy, 2002; Ikeda & Shimada, 2003; Newby & Zaltsman, 2000; Yang *et al.*, 2015).

During advanced atherosclerosis, pioneer SMCs can switch their phenotype towards the osteoblast-like state, also called osteogenic SMCs.

This phenotype switching is usually triggered by high extracellular phosphorus and calcium levels, oxidized lipoproteins and ROS. Osteogenic SMCs are the principal cause of vascular calcification as they release calcium phosphate enriched vesicles that are extruded into the lipid necrotic core, causing its calcification in advance plaques (Alves *et al.*, 2014; Hortells *et al.*, 2018; Sun *et al.*, 2012). Several transcription factors have been shown to be involved in the transformation of SMCs towards an osteogenic phenotype. These include KLF4, TLR4/NF-Kb, TGF-β/SMAD, RUNX2 (Runt-related transcription factor 2), and ERK1/2/ELK1/c-Fos (Farrokhi *et al.*, 2015; Goettsch *et al.*, 2011; Taylor *et al.*, 2011; Yan *et al.*, 2011). Among them, RUNX2, a transcription factor essential for bone formation, is considered an early expressed driver of the osteogenic transition of SMCs (Chen, Zhao and Wu, 2020). RUNX2 regulates several genes implicated in bone matrix production such as *COL1A1* (Collagen Type I Alpha 1 Chain), *SPP1* and *FN1* (Fibronectin 1) and also controls signalling pathways involved in osteoblast differentiation, as well as progenitor proliferation (Komori, 2019). Moreover, several signalling pathways such as the BMP2, ERK/MAPK, and PI3K/AKT appear to regulate the post-transcriptional level of RUNX2 and, therefore, control transcription of osteogenic genes via RUNX2 activity (Sun *et al.*, 2012). KLF4, which is found to be induced in calcified aorta, has been identified as a one of the main regulators of SMC transition towards an osteogenic phenotype and its increased expression contributes to the acceleration of plaque calcification and plaque destabilization (Alencar *et al.*, 2020).

Ultimately, the accumulation of cell debris originating from HASMCs and Mϕs, coupled with insufficient efferocytosis, provokes secondary necrosis from apoptotic cells which releases further molecules that have a pro-inflammatory effect, such as DAMPs (damage-associated molecular patterns) and the formation of a necrotic core within the plaque (Bäck *et al.*, 2019). This process culminates in the evolution of a plaque to a vulnerable state, characterized by an increased risk of rupture. Clinically, it is defined by a large necrotic core accompanied with a thin cap fibroatheroma. Development of fibroatheroma starts with the presence of calcified micro-

vesicles that will coalesce into larger speckles and fragments creating plates or sheets (Hutcheson *et al.*, 2016).

Later, the fragmentation of calcified sheets cause the formation of calcium phosphate rich nodules that can extend into the lumen of the vessel wall and trigger thrombosis, resulting in plaque rupture, dispersal of fragments through the circulatory system, that may cause myocardial infarction or stroke at distant sites if they become stuck and cause blockages in the vessels (Virmani *et al.*, 2000).

2.2.4 Immune cells

Immune cells belonging to both innate and adaptative immunity compartments account for a major part of the cellular landscape of an evolving atherosclerotic plaque.

Innate immune cells such as dendritic cells, mast cells and neutrophils, or the previously described monocytes and Mϕs, have been shown to play an important role in disease progression (Libby *et al.*, 2013). Moreover, recently, natural killer (NK) cells and other non-cytotoxic innate lymphoid cells have been studied for their involvement in the disease and have been detected in both mouse and human atherosclerotic lesions (Bonaccorsi *et al.*, 2019; Zernecke *et al.*, 2020). NK cells have been seen to promote lesion growth in mice though the production and release of perforin and granzyme which promote inflammation, as well as necrotic core formation (Nour-Eldine *et al.*, 2018). Regarding dendritic cells, their ability to modulate atherogenesis appears to originate from the regulation of T cell activation and responses in an antigen-dependant manner (Roy, Orecchioni and Ley, 2022).

On the other hand, the adaptative immune system, comprising of T cells, B cells, and natural killer T cells, has also been shown to be involved in atherosclerosis throughout the course of the disease. In the normal artery wall, the presence of resident dendritic cells has been shown to promote tolerization to antigen by silencing T cells. Nonetheless, proatherogenic stimuli activate dendritic cells which promote the activation of the adaptative immunity compartment (Niessner *et al.*, 2006; Niessner and Weyand, 2010).

T cells are located in the adventitia of arteries and are recruited into the lesion, alongside Mϕs, and in a similar fashion, exert their action via chemokines and adhesion molecules such as CCR5 (C-C chemokine receptor type 5) and CXCR6 (C-X-C chemokine receptor type 6), as previously discussed (Hansson, Robertson and Söderberg-Nauclér, 2006).

Before disease onset, regulatory T cells, which are critical for the maintenance of immune homeostasis, are the most abundant subtype found in the arterial wall. However, as the disease progresses, the pro-inflammatory CD4+ effector T cells outcompete other T cell populations and become the predominant subtype. Among them, IFNγ-secreting Th1 cells are the most prominent CD4+ effector T cell subtype. The secretion of IFNγ increases the engulfment of oxidized LDL, the subsequent foam cell formation and promotes the polarization of Mϕs towards a pro-inflammatory phenotype as well as stimulating the proliferation of SMCs (Saigusa, Winkels and Ley, 2020). In addition, Th2 cells have been described in the context of atherosclerosis and appear to carry an athero-protective function with the expression of cytokines such as IL-5 and IL-13 (Saigusa, Winkels and Ley, 2020). In regressing plaques, regulatory T-cells, through the activation of Mϕs, participate in the dampening of inflammation and tissue repair via improved efferocytosis (Sharma *et al.*, 2020). However, regarding their proportion in human atherosclerotic plaques, T cells are 10 times less abundant than Mϕs (Tedgui and Mallat, 2006).

Amongst the representatives of the adaptative immune system, B cells and mast cells are occasionally found in human plaques. Proliferation and activation of B cells has been identified as an important factor in the risk of CVD by genome-wide association and transcriptomic studies (Huan *et al.*, 2013). In atherosclerosis, B cells carry out a complex role as they can produce anti-inflammatory cytokines such as IL-10 but also pro-inflammatory cytokines such as GM-CSF (granulocyte- monocyte colony stimulating factor) and TNFα (Rosser and Mauri, 2015). Moreover, upon pro-atherogenic stimuli exposure, B-cells can differentiate into antibody-secreting plasmablast cells. These cells secrete immunoglobulins that have various functions. Amongst them, IgMs have the ability to neutralize oxidation specific epitopes present on OxLDL particles as well as on

apoptotic cell debris. Consequently, they have an athero-protective effect by limiting endothelial cell activation and foam cell formation (Chou *et al.*, 2009). Conversely, plasmablasts can also excrete IgE immunoglobulins which have strong pro-atherogenic properties attained through their binding to FcεRI on mast cells or Mϕs, leading to the former cell types activation (Tsiantoulas *et al.*, 2017). Mast cells appear to promote atherosclerosis through the production of pro-inflammatory cytokines, such as IL-6 and IFNγ, and auto-degranulation which leads to the release of histamine, which in turn, increases the accumulation of cholesterol in lipid-laden cells (Sun *et al.*, 2007; Wang *et al.*, 2011). In Mϕs, IgE exposure leads to increased secretion of IL-6 and apoptosis (Wang *et al.*, 2011). Similarly, plasmablasts secrete IgG as well, which binds to Fcγ receptors expressed on macrophages and this leads to the formation of immune complexes with OxLDL and the promotion of Mϕs inflammatory responses with the production of cytokines (Nimmerjahn and Ravetch, 2005; Ng, Burris and Nagarajan, 2011).

Finally, B cells and plasma cells can be found on the abluminal and adventitial side of the atherosclerotic artery, where artery tertiary lymphoid organs reside, during the advanced stages of plaque formation (Kovanen, 2007; Gräbner *et al.*, 2009).

2.2.5 Fibroblasts

Located in the adventitia of the aortic vessel, fibroblasts with a collagen and elastin rich ECM are arranged in a longitudinal network and are a driver of organ homeostasis and repair mechanisms in response to stress (Gladka *et al.*, 2018). During atherogenesis, the fibroblasts are a prevailing cell population and are mainly involved in the formation of the fibrous cap with the remodelling of the extracellular matrix and collagen production, with an additional role in the regulation of inflammation (Brokopp *et al.*, 2011). In the aortic vessel, fibroblasts consist of two subpopulations exhibiting a phenotypic gradient between them. Common fibroblasts markers include FAP, FSP1 (Fibroblast-Specific Protein 1), TCF21 (Transcription factor 21), PDGFRα (Platelet-Derived Growth Factor Receptor α), SCA1 (Stem Cell

Antigen-1) and lumican. However, these markers are not present in all fibroblast populations and are expressed by other cell types, rendering fibroblast identification complex (Kuwabara and Tallquist, 2017). Fibroblast heterogeneity appears to originate according to their different origin. Classically, fibroblasts have been thought to derive from mesenchymal-stem cells present in the adventitia of the tissue (Kramann *et al.*, 2016). However, in recent years, other cell-types appear to be the progenitors of this fibroblast population. As briefly mentioned before, a fibroblast population can derive from SMCs. Upon feeding of a high-fat diet, SMCs differentiate into fibromyocytes by decreasing their expression of SMC markers and shifting towards an increase in the expression of fibroblast markers (Wirka *et al.*, 2019). Another source of fibroblasts appears to be derived from ECs undergoing an endothelial-to-mesenchymal transitioning (endoMT). This metamorphosis involves the downregulation of endothelial associated genes like VE-cadherin (vascular endothelial cadherin), PECAM1, Tie-2 and CD31, which is followed by an upregulation of mesenchymal associated genes such as N-cadherin, FSP-1 (fibroblast-specific protein-1), α-SMA (α-smooth actin) and FAP (Goumans, van Zonneveld and ten Dijke, 2008). This endoMT transition is induced by stimuli such as hypoxia, TGF-β mediated signalling, oxidative stress and oscillatory shear stress (Evrard *et al.*, 2016; Lai *et al.*, 2018).

2.2.6 Other cell types

Several rare types of cells have been identified in the different layers of the aorta wall. Among them, neurons are believed to be play an important role in vessel contraction. A recent study has uncovered a complex crosstalk between arteries, the nervous system and the immune system in atherosclerosis, thus shedding light on a potential new approach for the prevention and treatment of cardiovascular diseases (Mohanta *et al.*, 2022).

2.3 ATHEROSCLEROSIS-LINKED PATHOGENIC STIMULI

Atherosclerosis is the result of a cascade of pathogenic stimuli including elevated blood cholesterol levels and hypertension, alongside unhealthy lifestyle factors, such as a lack of physical activity, obesity and the insidious influence of smoking. Altogether, these factors initiate vascular endothelial cell dysfunction, setting the stage for a chronic inflammatory response. This thesis will focus on two key stimuli mimicking the conditions that cells encounter during atherosclerosis, namely hypoxia, characterized by a decreased oxygen supply in advanced lesions, and oxidized lipids, which result from the free radical induced modification of regular lipids, that are incorporated in to low-density lipoproteins (LDL), and induce inflammation.

2.3.1 Oxidized Phospholipids

LDL has been identified as a cornerstone of atherosclerosis development and further clinical sequelae (Goldstein and Brown, 2015; Ference *et al.*, 2017). Given that LDLs are the most prevalent lipoprotein particles present in plasma, it serves as the primary conduit for cholesterol delivery within the arterial wall. LDL transcytosis is mediated by a vesicular pathway comprising of the LDL receptor (Zhang, Sessa and Fernández-Hernando, 2018), ALK1 (activin receptor-like kinase 1) (Kraehling *et al.*, 2016), and SR-B1 (scavenger receptor B1) coupled with DOCK4 (guanine nucleotide exchange factor dedicator of cytokinesis 4) (Armstrong *et al.*, 2015; Huang *et al.*, 2019). Interestingly, LDL transcytosis is inhibited by the downregulation of SR-B1 in presence of oestrogens, giving a potential explanation to why women have lower levels of atherosclerosis, compared to men, before menopause (Ghaffari *et al.*, 2018; Gordon, 1978; Sessa, 2018). However, subendothelial LDL accumulation and the subsequent development of atherosclerosis could also be explained by its selective retention in the arterial wall. This could be caused by the interaction of positively charged amino acyl residues on the APOB100 protein binding domain with negatively charged sulphate and carboxylic acid groups of proteoglycans on the arterial cells (Borén *et al.*, 1998). However, LDL levels themselves are not sufficient to explain the development of atherosclerosis (Zakiev *et al.*, 2016).

LDL retention in the intima triggers oxidation of LDL particles by a variety of different mechanisms, firstly, through enzymatic processes, driven by myeloperoxidase and lipoxygenases and secondly by non-enzymatic modification by reactive oxygen species. The result is the generation of oxidized LDL (OxLDL) particles containing several bioactive entities including oxidized phospholipids (Bochkov *et al.*, 2010; Binder, Papac-Milicevic and Witztum, 2016). The role of oxidized lipids in atherosclerosis has been recognized for a long time in humans (Quinn *et al.*, 1987) and was further confirmed by the discovery of the auto-antibody response to OxLDL in apolipoprotein E deficient mice (Palinski *et al.*, 1996). Amongst the modified phospholipids, the oxidation products of 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (oxPAPC) are the most abundant in human tissues (Berliner, Leitinger and Tsimikas, 2009). As described earlier, these oxidized LDL particles trigger a sterile inflammatory response through the activation of ECs and a subsequent upregulation of adhesion molecules and chemokines that act as signals for the recruitment of monocytes into the artery wall (Weber and Noels, 2011).

Several studies have shown that oxidized phospholipids incorporate into lipid membranes of cells and lipoproteins as well. In the cells, oxidized lipids act as ligands to cell membrane proteins or cause local membrane disruption (Morrow *et al.*, 1992; Moumtzi *et al.*, 2007). The uptake of oxPAPC into cells occurs via scavenger receptors such as CD36, SR-PSOX, and LOX-1. Alternatively, OxPAPC can enter cells by interacting with soluble factors and chaperones such as complement factor H, C3a, C-reactive protein, annexin 5, immunoglobulins and the toll-like receptors (Palinski *et al.*, 1996; Park, 2014). In Mϕs, the interaction between CD36 and OxLDL activates the Jun Kinases (JNK) 1 and 2 through phosphorylation of Lyn and this further increases the uptake of OxLDL (Park, 2014). Furthermore, CD36 promotes Mϕ differentiation into foam cells via the phosphorylation and subsequent activation of SRC (Src kinase) and the MAPK pathway. In addition, CD36 can trigger the formation of cholesterol crystals from endocytosed OxLDL which induces the production of IL1β by activation of the NLRP3-dependent inflammasome (Duewell *et al.*, 2010; Sheedy *et al.*, 2013).

Finally, oxidized phospholipid binding on toll-like receptors 2 or 4 activates signalling pathways which lead to the increased expression of chemo-attractant molecules such as CXCL8, CC-chemokine ligand 2 (CCL2) as well as fibronectin-containing connecting segment 1, and P-selectin. The presence of these molecules trigger the binding of monocytes to ECs, leading to their migration into the intima (Hartvigsen *et al.*, 2009; Kadl *et al.*, 2011; Binder, Papac-Milicevic and Witztum, 2016).

2.3.2 Hypoxia

Oxygen is an essential component for most species on earth. Hypoxia in biological systems is defined as a decreased oxygen concentration to the point where normal tissue homeostasis cannot be maintained. Hypoxia can be found in various pathological conditions such as cardiovascular disease, inflammation, cancer, and bacterial infection, for example. In cancer, hypoxia arises from the rapid proliferation of cancer cells, causing a demand for oxygen that outstrips available supply. In atherosclerosis, on the other hand, hypoxia occurs because of the accumulation of cells within the intima of the blood vessel that leads to a decrease in the availability of oxygen to the cells.

Hypoxia has been shown to regulate a plethora of genes, mainly induced by hypoxia inducible factors (HIFs) although other transcription factors such as Nuclear factor kappa B (NF-κB) are involved (Downes *et al.*, 2018; Taylor *et al.*, 2016). The HIF transcription factors are the main factors involved in the transcriptional response to hypoxia (Keith, Johnson and Simon, 2011). HIFs are heterodimers composed of a α protein subunit (HIFA and HIF2A), that is only stably expressed during hypoxia, and a β subunit, that is constitutively expressed. In normoxia, the HIF1α subunit is polyubiquitinylated by the von Hippel– Lindau (VHL) complex and degraded by the proteasome (Semenza, 2012). This phenomenon is controlled upstream by the prolyl hydroxylases (PHDs) that hydroxylate the two proline residues in the oxygen-dependent degradation (ODD) domain of HIFα (Kaelin and Ratcliffe, 2008; Semenza, 2012). Interestingly, the PHD enzymes are capable of hydroxylating both HIF1α and HIF2α. However, this process

requires oxygen, iron (Fe2+) and α-ketoglutarate. In a hypoxic environment, the hydroxylation of HIF1α or HIF2α and their further degradation through the proteasome machinery is interrupted, leading to their accumulation, dimerization with their partner proteins and translocation into the nuclei, where they bind the promoters of their respective target genes.

2.4 GENE REGULATION

Gene regulation is the process of controlling gene expression. It can be done by either turning genes on or off or fine-tuning gene expression that already exists. Gene regulatory mechanisms underlie cell identity through the control of cell-type specific gene expression and cellular responses to stimuli through activation or repression of genes which help cells to adapt to changes in their environments. Gene regulation is controlled at different steps, including transcriptional, post-transcriptional and translational levels. Ultimately, the interplay between all these mechanisms defines the functional output of a cell in healthy and disease states.

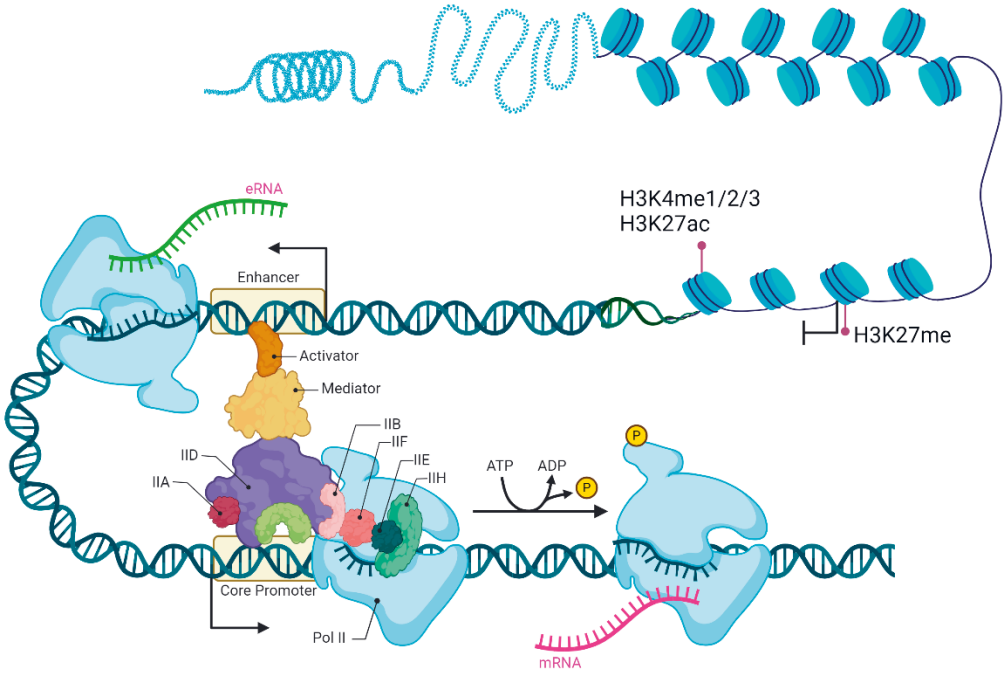

Figure 2: **Regulation of transcription in eukaryotic cells.** Chromosomal looping facilitates the interaction of distal enhancers with core promoter elements. Both enhancers and promoters are decorated with transcription

factors (TFs), RNA polymerase II (RNA pol II), the Mediator complex, and the basal transcriptional machinery. This coordinated assembly facilitates the initiation of transcription and subsequent RNA polymerase II mediated transcript elongation. IIA, IIB, IID, IIE, IIF, IIH: General transcription factors. Pol II: RNA Polymerase II.

2.5 TRANSCRIPTIONAL REGULATION

2.5.1 Transcription process

In eukaryotes, transcription is a complex process involving many actors (**Figure 2**). The process of transcription is carried out in sequential steps: initiation, pausing, elongation, and termination. In mammals, promoter-proximal pause-release occurs on more than 95% of genes (Jonkers *et al.*, 2014) and can coordinate transcriptional responses in stress and development (Adelman and Lis, 2012; Vihervaara *et al.* , 2018). There are three RNA polymerases (RNA pols) in eukaryotes. Whilst RNA pol I and III are dedicated to the transcription of rRNAs (except 5S rRNA) and small non-coding RNAs, including tRNAs (and the ribosomal 5S RNA), respectively, RNA pol II transcribes all mRNAs and miRNAs, as well as some other small RNAs such as snRNAs and siRNAs. Since RNA pol II transcribes mRNA and miRNAs, it is the focus of the following sections.

Transcription initiation occurs when the RNA pol II binds to the core promoter DNA, along with initiation factors and general transcription factors (GTFs), in order to form the pre-initiation complex (PIC) (Reinberg *et al.*, 1998; Grummt, 2003). Subsequently, the function of the PIC is to open the DNA strands. The opening of DNA by RNA Pol II is generally mediated by the DNA translocase XPB, a subunit of the TFIIH complex (Egly and Coin, 2011). XPB propels the template DNA strand into the RNA Pol II active site by separating the DNA strands in an ATP hydrolysis dependent process (Grünberg, Warfield and Hahn, 2012).

The elongation phase of transcription is tightly regulated. RNA Pol II often pauses and accumulates around 30-60bp downstream of the TSS (Rougvie and Lis, 1988; Strobl and Eick, 1992; Kwak *et al.*, 2013; Tome, Tippens and Lis, 2018). The pausing of the polymerase is a common regulatory process

involving promoter-associated transcription factors, the negative elongation factor (NELF) and the DRB-sensitivity-inducing factor (DSIF) that will stabilize the paused RNA pol II complex (Adelman and Lis, 2012; Kwak and Lis, 2013). The release of RNA Pol II from its promoter-proximal paused site is controlled by the positive elongation factor-b (P-TEF-b) complex consisting of cyclin T1 and cyclin-dependent kinase 9 (CDK9) (Peterlin & Price, 2006; Zhou *et al.*, 2012). P-TEF-b is recruited to promoters via direct or indirect binding involving specific TFs and co-factors such as the Mediator complex, the PAF1 complex and BRD4 (Lu *et al.*, 2016). Subsequently, P-TEF-b phosphorylates the carboxy-terminal domain (CTD) of RNA Pol II, NELF and DSIF leading to the eviction of NELF from RNA Pol II and the functional switching of DSIF to become a positive elongation factor (Peterlin and Price, 2006; Adelman and Lis, 2012; Kwak and Lis, 2013).

The productive elongation step begins after the release of RNA Pol II from its paused site. The transcription process is tightly coordinated with events leading to the proper processing of the RNA transcript (Perales and Bentley, 2009). Amongst them, nuclear RNA capping is the first modification to take place on nascent transcripts (Shatkin and Manley, 2000; Moteki and Price, 2002). The capping is carried out, firstly, with the removal of the 5′ γ-phosphate by the RNA TPase to generate 5′ diphosphate RNA transcript. Next, a GMP group is transferred to the 5′ diphosphate RNA molecule by Gtase. Later, a methyl group is added to the N7 amine of the guanine cap by the guanine-N7 Mtase. Finally, the methylation of the ribonucleotide by the m7G-specific 2′-O methyltransferase generates the mature methylated cap structure (Ramanathan, Robb and Chan, 2016). Productive elongation is also regulated by chromatin structure, histone modifications and the chromatin landscape.

During the elongation phase, introns are excised from the nascent RNA concurrently. This process is mediated by the spliceosome, a megadalton ribonucleoprotein molecular complex that assembles along each pre-mRNA intron, and catalysed by the small nuclear ribonucleoproteins (snRNPs) of which it is comprised (Kastner *et al.*, 2019). Components of the spliceosome recognize and bind to the consensus sequences present at the 5′ and 3′ end of introns. Then, two transesterification reactions will remove the intron and

covalently join the two adjacent exons. Amongst them, U1 binds to the consensus sequence in the 5′ end of introns. Then, the 5′ end of the intron base pairs with the sequence of the downstream branch of the intron which forms a looped structure called a lariat. Next, the 3′ end of the upstream exon is cleaved and the free OH group of this end attacks the phosphodiester bond at the 3' of the splice site by transesterification. Subsequently, the exons are bound covalently together and the lariat containing the intron is freed. Interestingly, when a 3′ splice site is suboptimal, alternative splicing often occurs. In such case, the suboptimal 3′ site is partially used in intron excision and exon ligation, leading to various proportions of mature mRNAs containing different exon combinations (Marasco and Kornblihtt, 2022). Alternative splicing has been observed in more than 90% of multi-exon genes and around 85% with a minor isoform frequency of more than 15%, making it is the most prominent mechanism for generating mRNA linear structural complexity and subsequently produces multiple protein isoforms, if translated (Jiang & Chen, 2021).

Transcriptional termination is defined as the release of the RNA pol II and the nascent transcript from the DNA template. Initially, the nascent transcript is cleaved by the cleavage and polyadenylation specificity factor (CPSF) 18–30 nucleotides downstream of the polyadenylation signal. After the cleavage, the nuclear 5′-3′ exonuclease Xrn2 is recruited to the polyadenylation site, where it progressively degrades the nascent transcript concurrently with the ongoing elongation carried out by RNA pol II. Xrn2 chases down RNA pol II, eventually catching up to it. Upon reaching RNA pol II, Xrn2 terminates transcription and releases RNA pol II from the DNA template (Connelly and Manley, 1988; Proudfoot, 1989, 2016).

Polyadenylation is controlled by *cis* elements, such as hexamers A[A/U]UAAA, U-rich or GU-rich elements, located upstream or downstream of the polyadenylation signal in a combinatorial manner (Cheng *et al.*, 2006). The poly adenylation machinery is comprised of nearly 20 core proteins arranged in four main complexes: CPSF (cleavage and polyadenylation specificity factor), CSTF (cleavage stimulation factor), CFI (cleavage factor I) and CFII (cleavage factor II) (Shi *et al.*, 2009). Before the cleavage of the nascent transcript, the polyadenylation signal is recognized by WD repeat

domain 33 and CPSF-30 subunits and cleaved by the endonuclease CPSF-73. Assemblage of those proteins around the pre-mRNA leads to the recruitment of PAP (poly(A) polymerase) that will synthesise the poly(A) tail on the transcript at the cleavage site (Tian and Manley, 2017).

Interestingly, between 50% and 80% of transcripts possess multiple poly(A) sites which leads to RNA isoforms with different length, coding sequences, and variable 3'UTRs allowing for differential availability of binding sites for RBPs and miRNAs (Derti *et al.*, 2012; Tian, 2005). Consequently, this heterogeneity of transcripts not only participates in transcriptome complexity but also plays an active role in determining RNA stability, localization, transport, half-life, as well as other functions. Moreover, alternative poly(A)s appear to be tissue/cell type specific and to be involved in multiple cellular processes such as cell proliferation, differentiation, and responses to stimuli (Arora *et al.*, 2022).

2.5.2 Promoters

In a nutshell, promoters are a sequence of DNA located at the 5' end of genes, upstream of their transcriptional start site (TSS) that allows the assembly of the transcriptional machinery with the binding of RNA polymerase II in order to initiate the transcription of DNA into RNA (Smale and Kadonaga, 2003).

The promoter core contains all the necessary components to allow the assembly of the transcriptional machinery such as the different subunits of the RNA Pol II and other multi-protein complexes such as TFIID. One of the key elements of the core promoter is the TATA box. The TATA box is a binding motif located around 30 bp upstream of the TSS. It is the binding site for the transcription factor TBP (TATA box binding proteins) and is the most studied element of the core promoter. The transcription factor TBP associates with 20 different TBP-associated factors (TAFs), amongst those, 13 of which form the TFIID complex. Later, the DNA envelops the complex in a way similar to a nucleosome, and will recruit other general transcription factors, such as TFIIA, B, E, F and H, and RNA Pol II to mobilize to the genomic locus and commence transcription.

It has long been thought that the TATA box was determinant in identification of the promoter core. However, it has recently been shown that nearly 90% of mammalian core promoters do not carry a functional TATA box. Interestingly, the initiator (Inr) element, encompassing the TSS, has been shown to be the functional equivalent of a TATA box. Its purpose appears to be to guide the formation of the basal transcriptional machinery complex, after being recognized by the TAF1 and TAF2 subunits of TFIID, thus determining the location of the start site and driving the activity of the proteins involved in transcription (Chalkley, 1999; Louder *et al.*, 2016). However, the Inr itself is present in only 50% of all genes (Lagrange *et al.*, 1998). 60% of TSS regions within the human genome are located in the vicinity of CpG dinucleotide (CpG) islands. This defines another class of core promoter which have a high GC content and a lack of TATA box or Inr elements. These have been found to harbour multiple binding sites for the ubiquitously expressed mammalian sequence-specific transcription factors (SSTFs) such as SP1 (specificity protein 1) and E2F (Nguyen *et al.*, 2016). SP1 in combination with an Inr could function in lieu of a TATA box (Vo ngoc *et al.*, 2017) and guides the formation of the transcription machinery through the binding of TAF1, TAF2 and TAF4 and members of the E2F family which carry a binding site similar to the one of components of the basal transcriptional machinery. Interestingly, at promoters, bidirectional transcription has been observed (Core, Waterfall and Lis, 2008; Seila *et al.*, 2008; Core *et al.*, 2014). Usually, the transcription of protein-coding genes coincides with the transcription of short non-coding RNAs from the reverse orientation. These antisense transcripts are transcribed by distinct RNA pol II complexes from divergently oriented TSSs situated at the upstream edge of the promoter region. (Duttke *et al.*, 2015; Scruggs *et al.*, 2015).

The last decade has seen the growth of investigations on chromatin architecture of transcriptional promoters in yeast, fly and mammalian genomes using chromatin immunoprecipitation (ChIP) combined with either microarrays (ChIP on a chip) or sequencing (ChIP-seq) of DNA fragments which were bound by a variety of histone tail residue modifications, RNA pol II, and transcription factors (Barrera and Ren, 2006). These studies have shown that active promoters are discernible through acetylation of various

residues of histones H3 and H4 and that these mechanisms are conserved across species (Heintzman *et al.*, 2007). H3K4me3, for example, is not only a marker for active promoters but is also seen in poised TSS regions where RNA pol II is stalled.

2.5.3 Enhancers

Enhancers are short DNA sequences that are found either upstream, downstream or within the genes they regulate and therefore, opposed to promoters, they can act at a distance and in an orientation independent fashion (Blackwood and Kadonaga, 1998; Bulger and Groudine, 1999). Enhancers are often considered as the driving force of cell-type specific gene expression. Most enhancers have shown a tightly restricted temporal and spatial window of activity (Nord *et al.*, 2013). Generally, enhancer regions are marked by an enrichment for histone 3 lysine 4 mono or di-methylation (H3K4me1/2) and histone 3 lysine 27 acetylation (H3K27Ac), along with lower enrichment of H3K4me3 compared to promoters, alongside nucleosome depletion and the synthesis of short enhancer RNAs correlating with their activity (Heintzman *et al.*, 2007; Creyghton *et al.*, 2010; Hnisz *et al.*, 2013; Kaikkonen *et al.*, 2013).

The human genome has been shown to be comprised of nearly 668 000 putative enhancer-like sequences, which represent 5.6% of the whole genome (ENCODE Project Consortium *et al.*, 2020). Considering that the number of putative enhancer is far greater than the number of genes, it became apparent than the control of a single gene involved multiple enhancers (Long, Prescott and Wysocka, 2016). Consequently, several studies have found that most individual enhancers can be inactivated without serious repercussions to a target gene's expression (Frankel *et al.*, 2010; Montavon *et al.*, 2011; Fulco *et al.*, 2016). This enhancer redundancy has a critical role as it allows to partially compensate for the loss of other enhancers but also to smother the effect of genetic differences or environmental stress (Osterwalder *et al.*, 2018).

When enhancers are clustered into large regions containing multiple lineage specific TFs binding sites, the components of the transcriptional

machinery and histone modifications such as H3K27ac, these regions are called super-enhancers (Whyte *et al.*, 2013). These super-enhancers have been shown to be enriched in close proximity of key cell-type specific genes (Whyte *et al.*, 2013; Heinz *et al.*, 2015) and play a key role in cellular response to stimuli (Brown *et al.*, 2014; Hah *et al.*, 2015). Several studies have examined the role of enhancers that are a part of a super-enhancers. Interestingly, they showed that those enhancers have additive and redundant relationship. Moreover, deletion of one enhancer caused a reduction of activity of other enhancer elements including histone acetylation, transcription factor binding, enhancer RNA production as well as altering both the enhancer-promoter and enhancer-enhancer interaction interplay (Huang *et al.*, 2016, 2018; Jiang *et al.*, 2016; Proudhon *et al.*, 2016; Shin *et al.*, 2016).

Some enhancer sequences are transcribed by RNA polymerase II to form enhancer RNAs (eRNAs). In human cells, around 40,000–65,000 eRNAs have been reported (Andersson *et al.*, 2014; Arner *et al.*, 2015). The eRNAs are short, bi-directionally transcribed and non-polyadenylated non-coding RNAs. Interestingly, several studies have reported that an eRNAs expression level is correlated with enhancer activity (Andersson *et al.*, 2014; Lai *et al.*, 2015; Lam *et al.*, 2013; Li *et al.*, 2013). Furthermore, eRNAs were shown to control the expression of nearby protein-coding genes in an RNA-dependent manner. In addition, enhancers producing eRNAs have been shown to display higher transcriptional co-activator binding, increased chromatin accessibility and enrichment of active chromatin associated histone marks such as H3K27ac, as compared to enhancers which do not produce them (Hah *et al.*, 2013; Kaikkonen *et al.*, 2013; Kim *et al.*, 2010; Melgar *et al.*, 2011; Zhu *et al.*, 2013). Furthermore, eRNA-producing enhancers have also been shown to participate in transcriptional regulation. For example, eRNAs can take part in transcriptional interference in *cis* through the displacement of transcription factors or other binding proteins during RNA Pol II mediated transcript elongation. Additionally, RNA pol II transcription of eRNAs can reposition nucleosomes and thereby occlude gene promoters or other DNA-binding protein binding sites. Eventually, during productive transcription, RNA Pol II synthesizing an eRNA can clash with another RNA Pol II

synthesizing mRNA in the other direction, leading to a collision of polymerase complexes, their destabilisation, and transcription termination (Hobson *et al.*, 2012).

Conversely, eRNA transcription can induce gene transcription in *cis* through the displacement of transcriptional repressors such as Polycomb group (PcG) proteins or nucleosomes, rendering promoter regions more accessible (Schmitt, Prestel and Paro, 2005; Maeda and Karch, 2015). Furthermore, eRNA transcription can modify the transcriptional landscape, such that RNA pol II elongation can spread active histone marks such as H3K4me1/2/3 across a wider region (Kaikkonen *et al.*, 2013; Kim *et al.*, 2016).

In addition, nascent eRNAs can retain TFs locally through short-lived interactions, increasing their effective concentration within the enhancer/promoter domain (Sigova *et al.*, 2015). Alternatively, eRNAs have been shown to increase local acetylation through binding of the histone acetyltransferase domain of CBP/P300 which stimulates this co-activator's activity (Bose *et al.*, 2017). Nascent eRNAs can also bind repressive histone mark complexes such as PRC2 inhibiting their ability to deposit repressive me3 onto H3K27 (Wang *et al.*, 2017). Moreover, eRNAs have been seen to associate with cohesin and the Mediator complexes that participate in the task of the formation of enhancer-promoter loops that regulate gene expression by increasing the concentration of transcriptional machinery elements nearby the promoters of genes (Sanyal *et al.*, 2012; Kaikkonen and Adelman, 2018).

2.5.4 Transcription factors

A requirement for an enhancer's activity is transcription factor binding. Transcription factors (TFs) are proteins regulating gene expression. These proteins are called transcription factors because of their ability to bind a DNA sequence in a sequence specific manner and to regulate the transcriptional process (Fulton *et al.*, 2009; Vaquerizas *et al.*, 2009). To date, the human genome has been found to encode over 1600 TF genes (Lambert *et al.*, 2018; Pratt *et al.*, 2022). Typically, TFs contain a DNA-binding domain (of different classes) of 60 to 90 amino-acids able to recognize 6 to 20 bp

long consensus DNA motif sequences present in the genome. Due to the large size of the human genome, this implies that most TFs have millions of potential binding sites, considering the frequency of small sequences occurring amongst the billions of base pairs.

TFs can be activators or repressors of gene transcription. A TF's activity is determined by its binding to accessible promoters, enhancers or other *cis*-regulatory elements (Istrail and Davidson, 2005). When acting as activators, TFs bind to *cis*-regulatory elements and recruit chromatin remodelling proteins that will open the chromatin and render the region accessible for the recruitment of the multi-subunit Mediator complex that is necessary for the activity of the RNA polymerase II (Blazek, Mittler and Meisterernst, 2005). On the contrary, if a transcription factor carries repressive function, it interferes with transcriptional machinery recruitment to the TSS or recruit chromatin modifiers that will create regions of repressive chromatin marked nucleosomes.

Interestingly, even though TFs can be found at any distance from their regulatory elements, the likelihood of a TF to be involved in the regulation of a gene decreases proportionately when the distance between the TF and the gene increases. However, it is worth noting that the most important parameter for TFs in its ability to regulate a given gene is the distance based on the 3D genome conformation and the membership to the same topologically associated domain (Stadhouders, Filion and Graf, 2019).

Transcription factors are categorized into two main categories: the constitutively active TFs (CATFs) and the signal or ligand- dependent TFs (S/LDTFs). The group of constitutively active TFs, also called "pioneer factors" or lineage determining transcription factors, is comprised of ubiquitous TFs that are found in the nucleus and involved in the regulation of housekeeping genes and cell-type specific TFs that are involved in cell-type determination, maintenance and differentiation (Zaret *et al.*, 2008; Heinz *et al.*, 2010). On the other hand, signal-dependent transcription factors (S/LDTFs) also called stimuli-specific transcription factors, constitute the largest group of TFs. These TFs are inactive or minimally active, until the cell is exposed to a specific stimulus that can be sensed intra or extra-cellularly. Some transcription factors bind their activating ligands directly, whilst others are

induced into activity indirectly by intracellular signalling pathways that are responsible for sensing the signal in the first place. (Mullen *et al.*, 2011; Samstein *et al.*, 2012).

Many transcription factors have been shown to be involved in diseases. However, for the different cell-type specific responses in any disease condition, the culprit is the interplay between the constitutively active and the signal/ligand dependent TFs. More specifically, studies have shown that constitutively active TFs can participate in nucleosome remodelling, leading to histone modification and ultimately establishing a new local epigenetic signature (Ghisletti *et al.*, 2010; Heinz *et al.*, 2010). This transition from closed chromatin to a primed or poised state initiated by CATFs allows for the binding of stimuli-dependent transcription factors to the region (Heinz *et al.*, 2010). Interestingly, there is a hierarchical relationship between CATFs and S/LDTFs as the loss of function of CATFs impairs the binding of both CATFs and S/LDTFs while the converse is not true as the loss of function of S/LDTFs does not hinder the binding of CATFs to the enhancer region (Heinz *et al.*, 2010; Mullen *et al.*, 2011; Sullivan *et al.*, 2011; Kaikkonen *et al.*, 2013).

2.6 NON-CODING RNA

One of the early fundamental principles of molecular biology was that RNA is transcribed to act as a template used by ribosomes for protein translation. However, with the development of sequencing techniques and the sequencing of the whole transcriptome and genome, it has been revealed that a majority of transcripts are never translated into protein (Carninci *et al.*, 2005; Derrien *et al.*, 2012; Hangauer, Vaughn and McManus, 2013). It has also been discovered that among the nearly 250 000 transcripts that can be transcribed in the cell, more than 60% are non-coding RNAs, with less than 2% being precursors for microRNAs (Frankish *et al.*, 2021).

Of note, but out of the scope of this thesis, miRNAs and lncRNAs have been found to be exported form the cells with the use of extracellular vesicles such as exosomes, micro-vesicles and apoptotic bodies, highlighting their importance as mediators of intercellular communication and potential

candidates for therapy and prognosis of disease. (Mori *et al.*, 2019; Zhang *et al.*, 2020).

2.6.1 Long non-coding RNAs

Amongst non-coding RNAs, a category has been drawing a lot of attention in the recent years are the long non-coding RNAs (lncRNAs). They are defined as transcripts of more than 200 nucleotides that are not translated into proteins. This size cut-off has been widely accepted as it is convenient in biochemical and biophysical RNA purification protocols. Moreover, due to this size cut-off, the lncRNA category does not include most infrastructural RNAs, such as small nuclear RNA (snRNAs), small nucleolar RNAs (snoRNAs), transfer RNA (tRNAs), 5S ribosomal RNA (5S rRNAs), small interfering RNA (siRNAs) and piwi-interacting RNA (piRNAs) that are all smaller than 200bp (Mercer, Dinger and Mattick, 2009).

So far, more than 53 000 lncRNAs transcripts have been identified (Frankish *et al.*, 2021). As with mRNAs, lncRNAs are mostly generated by RNA Pol II, are multi-exonic, highly alternatively spliced and polyadenylated. Conversely, lncRNAs generally display less well defined 3'-end processing. Their nomenclature has been decided based on their genomic location and orientation in respect to protein-coding genes as they can be transcribed from intergenic regions (lincRNAs), intronic regions or originate from sense or antisense transcripts overlapping other genes (Derrien *et al.*, 2012; Wright, 2014; Mattick and Rinn, 2015; Montes and Lund, 2016; Frankish *et al.*, 2021).

Interestingly, lncRNAs are less evolutionarily conserved than their mRNA counterparts. Long non-coding RNAs have been shown to change more rapidly over evolutionary time and across different species since they do not have a rigid structure to function relationship compared to protein-coding sequences and are consequently more affected by positive selection during adaptative radiation (Pheasant and Mattick, 2007; Quinn *et al.*, 2016). Many lncRNAs are also associated with developmentally active and cell lineage specific enhancers implying that their sequence variation constitutes a major factor in species biodiversity.

With respect to their relative expression, lncRNAs are often found at lower levels, compared to protein-coding gene transcripts, and are often highly cell type specific (Flynn and Chang, 2014; Gloss and Dinger, 2016). LncRNAs have also been shown to have specific subcellular location patterns even though they are more often enriched in the nucleus (Mas-Ponte *et al.*, 2017a). LncRNAs are also dynamically expressed and this expression is greatly affected by exogenous stimuli (Lakhotia, 2012; Barth *et al.*, 2020; Connerty, Lock and de Bock, 2020).

It has been hypothesized that lncRNAs carry out a role in regulating the transcription of genes in either *cis* or *trans*, the organisation of sub-nuclear domains, and regulation of proteins or RNA molecules (Ulitsky *et al.*, 2011).

Several studies have highlighted the role of lncRNAs in the regulation of multiple genes. In some instances, lncRNAs have been seen influencing the expression output of multiple genes in close proximity. This mechanism is driven by the interactions between lncRNAs and RNA-binding proteins, chromatin or transcription factors and the subsequent accumulation of those at the transcriptional units of genes (Joung *et al.*, 2017). In numerous cases, they have been revealed to be participating actively towards the recruitment of proteins to nearby genomic loci (Deng *et al.*, 2016; Li *et al.*, 2012; Liu *et al.*, 2016; Wang & Dostie, 2016). Additionally, the transcription of lncRNA itself can affect the expression of nearby genes without necessarily involving the lncRNA that is transcribed at this particular locus (Engreitz *et al.*, 2016). Furthermore, some studies have reported the binding of lncRNAs at multiple sites within regulatory elements, highlighting their role in the control of gene expression locally and distally (Vance *et al.*, 2014).

Due to their role in gene regulation through binding to RNA molecules or proteins, lncRNAs have been shown to play a role in many cellular processes such as cell stress response, cellular proliferation, cell migration, differentiation and senescence (Audas & Lee, 2016; Chen *et al.*, 2017; Grammatikakis *et al.*, 2014; Jingqiu Li *et al.*, 2016). Indeed, by acting on those cellular processes, lncRNAs have been shown to play a major role in various diseases such as cancers or cardiovascular diseases and are considered to have promising potential to be either prominent diagnostic and/or

therapeutic targets (Greco, Gorospe and Martelli, 2015; Schmitt and Chang, 2016; Alvarez-Dominguez and Lodish, 2017).

2.6.2 Function of lncRNAs

Long non-coding RNAs are located in various cellular compartments, however, the vast majority of them appear to reside in the nucleus (Derrien *et al.*, 2012; Djebali *et al.*, 2012). However, over the past years, it has become clearer that lncRNAs have different functions, depending on their subcellular localization (**Figure 3**). The follow sections will discuss these roles.

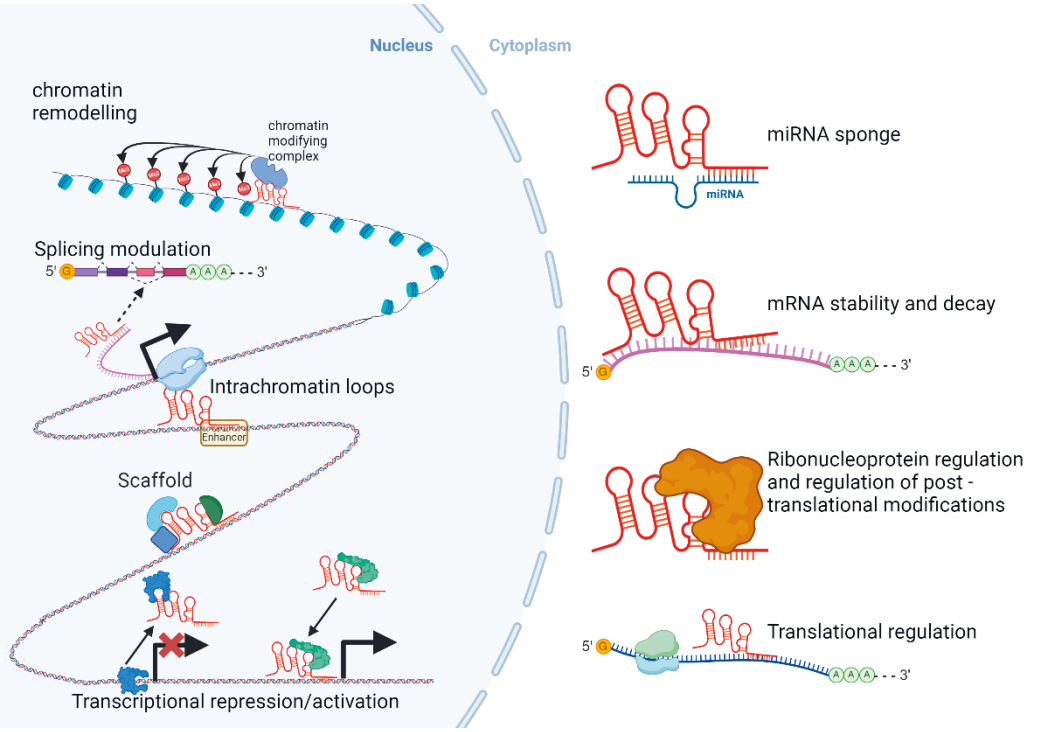

Figure 3: **The roles of lncRNAs in the different sub-cellular compartments.** In the nucleus, lncRNAs act as key regulators of gene expression. They interact with chromatin modifiers, promoting chromatin remodelling and altering the accessibility of DNA for transcription. Additionally, they influence chromosomal architecture and interactions, facilitating communication between distant genomic elements. Furthermore, lncRNAs can directly regulate gene transcription by interacting with the transcriptional machinery or through their own transcription and the associated DNA elements. Notably, some lncRNAs also influence mRNA

splicing events. Within the cytoplasm, lncRNAs contribute to the regulation of mRNA stability and decay. They can act as "miRNA sponges" sequestering these microRNAs and preventing their interaction with target mRNAs. Long non-coding RNAs can also interact with ribonucleoprotein complexes and participate in translational regulation, impacting the efficiency of protein synthesis. Moreover, lncRNAs are implicated in the coordination of post-translational protein modifications, further influencing cellular processes.

2.6.3 Nuclear lncRNAs

Amongst the nuclear-enriched lncRNAs, a substantial amount has been found to be associated with chromatin and have been classified as chromatin-enriched RNAs (cheRNAs) (Werner and Ruthenburg, 2015).

Nuclear lncRNAs can modify chromatin architecture by establishing three-dimensional infrastructures. This 3D scaffold can prevent chromatin condensation and maintain gene activity. Interestingly, lncRNAs can form chromatin loops over several megabase pairs that consist of spatially and functionally delimited concentration gradients of the ncRNAs themselves as well as other RNAs and proteins (Quinodoz *et al.*, 2021a). These 3D structures could be maintained by lncRNAs physically attached to, or in 3D proximity to their DNA loci such as cardiovascular disease associated lncRNAs *Airn*, *Kcq1ot1* or *MALAT1* (Quinodoz *et al.*, 2021b). Moreover, nuclear lncRNAs can modify chromatin architecture by enhancing the recruitment of and the association of chromatin-modulating proteins to the chromatin such as SWI/SNF (Switch/sucrose nonfermentable) and PRC (Polycomb repressive complex) subunits (Scarola *et al.*, 2015; Wang *et al.*, 2015). Conversely, nuclear lncRNAs can impede the association of chromatin-modulating proteins to their targeted gene loci. In this respect, a study of 3289 lincRNAs expressed in human cells have showed that nearly 25% are associated with PRC2 whilst with mRNAs, it was less than 2% (Khalil *et al.*, 2009).

Nuclear lncRNAs have also been shown to prevent histone deacetylase, methyl transferase and chromatin remodelling complex interactions to their specific genomic loci by acting as a decoy (Fan *et al.*, 2015; Han *et al.*, 2014; Jain *et al.*, 2016; Prensner *et al.*, 2013). Conversely, the pioneer transcription

factor CREBBP (CREB binding protein) has been seen to increase histone acetylation and gene transcription after binding to eRNAs (Bose *et al.*, 2017). Additionally, nuclear lncRNAs can exert their influence on chromatin organisation indirectly by forming interactions with chromatin modulators through other kinds of proteins (Xing *et al.*, 2014).

Nuclear lncRNAs can modulate transcription, either positively or negatively, and either *in cis* (locally or adjacently) or in *trans* (distally). Long non-coding RNAs have been shown to facilitate the recruitment and, therefore, the transcriptional activation of transcription factors (Liu *et al.*, 2017). They can also act as suppressors since some lncRNAs were found to repress mRNA expression by participating in the formation of a RNA-DNA triplexes which resulted in the recruitment of a transcriptional repressor to the target gene locus (O'Leary *et al.*, 2015). Some lncRNAs have also been shown to have the ability to sequester transcription factors, inhibiting or augmenting the latter's intended activity so that the lncRNAs would behave as transcriptional repressors or activators, respectively (Krawczyk & Emerson, 2014; Li *et al.*, 2017). Moreover, lncRNAs have been shown to regulate transcription factor activity by influencing their sub-cellular or sub-nuclear localization (Cecilia *et al.*, 2016; Lu *et al.*, 2014).

Furthermore, RNA-binding proteins (RBPs), a class of proteins that interact with either single or double-stranded RNA, have the potential to bind to nuclear-enriched lncRNAs and thus regulate their activity. Several studies have reported that the interaction between RBPs and nuclear enriched lncRNAs could facilitate interactions between TF and its bound RBP by acting as a scaffold in order to activate transcription (Huang *et al.*, 2015; Li *et al.*, 2014). On the other hand, lncRNAs bound to RBPs can curb transcription by interacting with transcriptional repressors (Bao *et al.*, 2015).

Some studies have uncovered the role of nuclear lncRNAs in the post-transcriptional regulation of genes. To this end, lncRNAs have been reported to promote cell-type specific alternative splicing by regulating the processing of pre-mRNAs (Gonzalez *et al.*, 2015). Post-transcriptional regulation through lncRNAs can also be achieved via the sequestration of RNA-binding proteins (Wu *et al.*, 2016).

Finally, it has been reported that lncRNAs located in the nucleus exert their regulation of gene expression through the reorganization of nuclear structure. Reshaping of the 3D structure of the chromatin, directed by lncRNAs, has been described by the binding of lncRNAs to proteins either to create Barr-bodies (which are inactivated X chromosomes in cells that possess two or greater X chromosomes, formed to correct for gene dosage effects on sex chromosome genes in female mammals) in order to repress transcription or to establish a "scaffold" to increase inter-chromosomal interactions (Chen *et al.*, 2016; Hacisuleyman *et al.*, 2014).

2.6.4 Cytoplasmic lncRNAs

In the cytoplasmic compartment, lncRNAs have been observed in several organelles such as mitochondria (Dong *et al.*, 2017), extracellular membranes (Lin *et al.*, 2017), ribosomes (Carlevaro-Fita *et al.*, 2016; Ingolia *et al.*, 2011; van Heesch *et al.*, 2014; Zeng *et al.*, 2018) as well as extracellular entities produced by cells, such as exosomes (Gezer *et al.*, 2014). It may seem peculiar that lncRNAs bind ribosomes, as they do not possess translated ORFs, this ribosome-lncRNAs interaction could be a sign of either undiscovered very short, but translatable, ORFs (Derrien *et al.*, 2012; Ruiz-Orera *et al.*, 2014) or an indication of other processes such as the degradation (Zeng, Fukunaga and Hamada, 2018) and regulation of ribosome bound mRNAs translation (Yoon *et al.*, 2012) or even off-target trafficking of capped and polyadenylated lncRNAs (Guttman *et al.*, 2013).

Cytoplasmic non-coding RNAs have been shown to modulate the turnover and translation of specific mRNAs. This mechanism is mediated through the binding of lncRNAs to the mRNA that recruits proteins that promote mRNA decay, as well as to supress or initiate translation. This modulation of mRNA stability can also be achieved through the hindrance of the binding of microRNAs to their target mRNAs (Gong & Maquat, 2011; Hu *et al.*, 2014; Yoon *et al.*, 2012). Cytoplasmic lncRNAs can also control protein degradation pathways by preventing or enhancing the availability of the mRNA through binding to various RNA stability process associated RBPs which subsequently alter the availability of the protein product of the mRNA

to the ubiquitin/proteasome machinery (Jun *et al.*, 2016; Yang *et al.*, 2014; Yoon *et al.*, 2013). Additionally, lncRNAs located in the cytoplasm have been shown to serve as a decoy for RNA binding proteins. This mechanism is performed by dissociating RNA binding proteins from the target mRNA, thus modulating the abundance of mRNA and it's translation (Buratti, 2008; Kim *et al.*, 2016, 2017; Liu *et al.*, 2012). Importantly, the binding of lncRNAs to RBPs in the cytoplasm reduces the availability these proteins in other sub-cellular compartments (Mourtada-Maarabouni *et al.*, 2009). Finally, cytoplasmic lncRNAs have also been shown to act as fine tuners of miRNA dependent translational repression. As such, lncRNAs carry out the role of competing with endogenous RNAs by reducing the accessibility of miRNAs to their target mRNAs (Kallen *et al.*, 2013; Tang *et al.*, 2016; Wang *et al.*, 2010).

2.6.5 microRNAs

One of the most recent revelations of modern biology was the discovery by the Ambros and Ruvkun groups of the first miRNA *lin4* in *Caenorhabditis elegans* (Lee *et al.*, 1993; Wightman *et al.*, 1993). From this discovery, up to now, nearly 40,000 miRNAs have been identified in 271 organisms. MicroRNAs are non-coding RNA sequences of 21-23 nucleotides that act as post transcriptional regulators on a wide variety of biological processes ranging from embryogenesis to disease development.

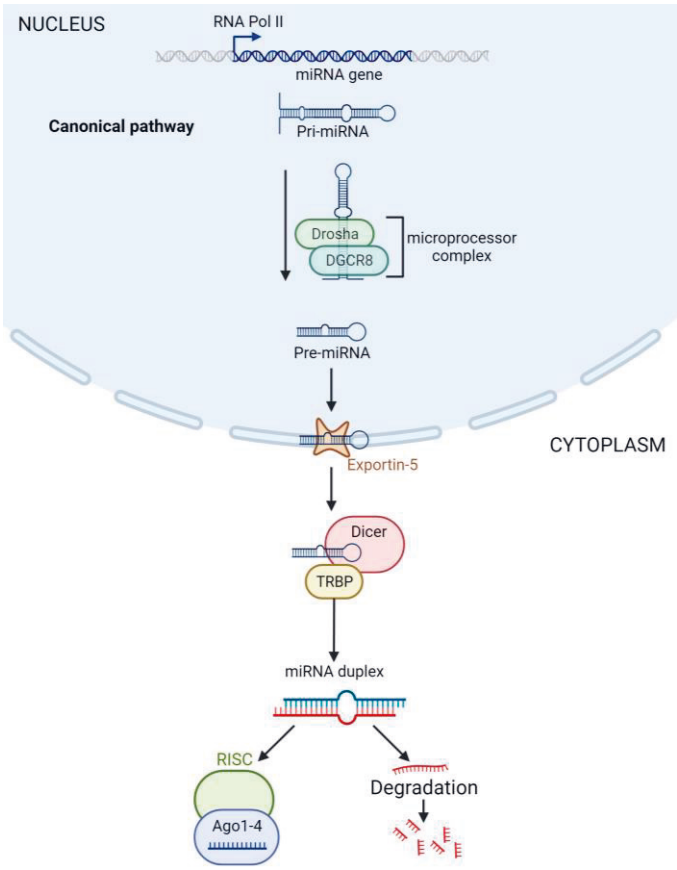

Figure 4: **miRNA biogenesis pathway.** In the nucleus, RNA polymerase II transcribes primary miRNA transcripts (pri-miRNAs). The microprocessor complex, consisting of DROSHA and DGCR8, cleaves pri-miRNAs to generate precursor miRNAs (pre-miRNAs). Exportin 5 facilitates the transport of pre-miRNAs to the cytoplasm. Within the cytoplasm, Dicer, in conjunction with its partner protein TRBP, processes pre-miRNAs into a double-stranded miRNA duplex. An Argonaute (AGO) protein recognizes and selectively binds to one mature miRNA strand from the duplex, forming the RNA-induced silencing complex (RISC). This mature miRNA-RISC complex is a key effector for post-transcriptional gene silencing, regulating gene expression.

2.6.6 Biogenesis of miRNAs

The biogenesis of microRNA begins in the nucleus with the transcription of a primary miRNA (pri-miRNA) transcript by RNA polymerase II (**Figure 4**). Pri-miRNA transcript sizes can be hundreds of kilobases in length (Cai, Hagedorn and Cullen, 2004; Lee *et al.*, 2004) and possess the features of coding mRNAs, such as capping, stem-loops and polyadenylation. Furthermore, pri-miRNA can contain more than one mature miRNA sequence (Lee *et al.*, 2002). Simultaneously or rapidly after the transcription, the stem-loop structures of primary microRNA are recognized by the microprocessor complex. The microprocessor complex is constituted of two nuclear proteins: the RNase III enzyme DROSHA and DGCR8 (DiGeorge syndrome critical region 8). DROSHA is a 159kDa protein located mostly in the nucleus that acts as the core of the microprocessor complex whereas DGCR8 is a nuclear protein of 86kDa which acts as an essential cofactor for DROSHA (Denli *et al.*, 2004; Gregory *et al.*, 2004; Lee *et al.*, 2003). Pri-miRNAs contain stem-loop structures of 60-80 nucleotides that are recognized and excised by the microprocessor complex. While DROSHA binds to the double-strand RNA–single-strand RNA junction at the hairpin base, two DGCR8 proteins recognize the stem and ensure cleavage of the pri-miRNA at the correct position (Kwon *et al.*, 2016; Nguyen *et al.*, 2015). These excised stem-loops are referred to as precursor miRNAs (pre-miRNAs) and contain the mature miRNA sequences. Pre-miRNAs contain a short sequence of 22 base pairs and a 3’ overhang of 2 nucleotides that is recognized by the nuclear export factor exportin 5. This causes the transportation of the pre-miRNA to the cytoplasm (Bohnsack *et al.*, 2004; Lund *et al.*, 2004; Yi *et al.*, 2003; Zeng & Cullen, 2004). In the cytoplasm, the endonuclease DICER recognizes the 5’ phosphate, the 3’ overhang and the loop structure of the pre-miRNA (MacRae *et al.*, 2007; Park *et al.*, 2011) and removes the terminal loop, generating a double stranded 18-24 nucleotide long RNA duplex (Hutvágner *et al.*, 2001; Ketting *et al.*, 2001) with a 2 nucleotide 3’ end overhang on each end of the duplex (Nicholson, 2014; Zhang *et al.*, 2004).

The next step in miRNA biogenesis is the RNA-induced silencing complex (RISC) loading, where the miRNA duplex is loaded onto ARGONAUTE

proteins. In humans, four ARGONAUTE proteins are expressed (AGO 1-4) and differ only by a few amino acids in their functional domain (Müller, Fazi and Ciaudo, 2020). Amongst them, AGO2 is the most abundant (Wang *et al.*, 2012) and possesses two motifs in its N-terminal domain required for its full catalytic activity. However, it has been shown that AGO1 contains only one of the two motifs required for the full catalytic action (Faehnle *et al.*, 2013; Hauptmann *et al.*, 2013) while AGO3 and AGO4 contain none (Nakanishi *et al.*, 2013; Park *et al.*, 2019; Schürmann *et al.*, 2013).

Next, the AGO protein loaded with miRNA duplex will select one strand to become the mature miRNA and discard the other. This step is usually referred to as "passenger ejection" as AGO expels the passenger strand of the miRNA duplex and forms the RISC complex with the remaining guide strand. Amongst the two types of "passenger ejection" methods, the most common one is the "slicer-dependent" one as it relies on the catalytic abilities of AGO proteins (Leuschner *et al.*, 2006). On the other hand, the alternative method is "slicer-independent" and is regulated by the thermodynamic stability of the RNA duplex (Gu *et al.*, 2011; Yoda *et al.*, 2010). Once the passenger strand is discarded, the RISC complex becomes the effector complex for RNA silencing.

Functional strand selection is a regulated process as cells are able to change their functional strand preferences under different conditions such as disease status or exposure to exogenous signals (Schober *et al.*, 2014; Guixé-Muntet *et al.*, 2017; Santovito *et al.*, 2020; Moreau *et al.*, 2021). However, this process involves post-transcriptional mechanisms that require further investigation, but does not implicate changes in transcription of pri-miRNAs which tends to be minimally affected by exogenous stimuli (Moreau *et al.*, 2021).

2.6.7 Function of miRNAs

Micro RNAs are involved in the regulation of many cellular process and their expression is often considered tissue or, more precisely, cell-type specific (Landgraf *et al.*, 2007). Besides, specific miRNAs profiles have been observed in different diseases and have been found to correlate with clinical

outcomes (Lawrie *et al.*, 2009; Esteller, 2011). One of the main roles of miRNA is post-transcriptional gene silencing via RISC complex action in the cytoplasm. However, recent studies have detected the presence of miRNAs in the nucleus, where it is suggested that they exert different functions (Liu *et al.*, 2018). Thus miRNAs, as is the case with lncRNAs, have different functions based on their sub-cellular location (**Figure 5**).

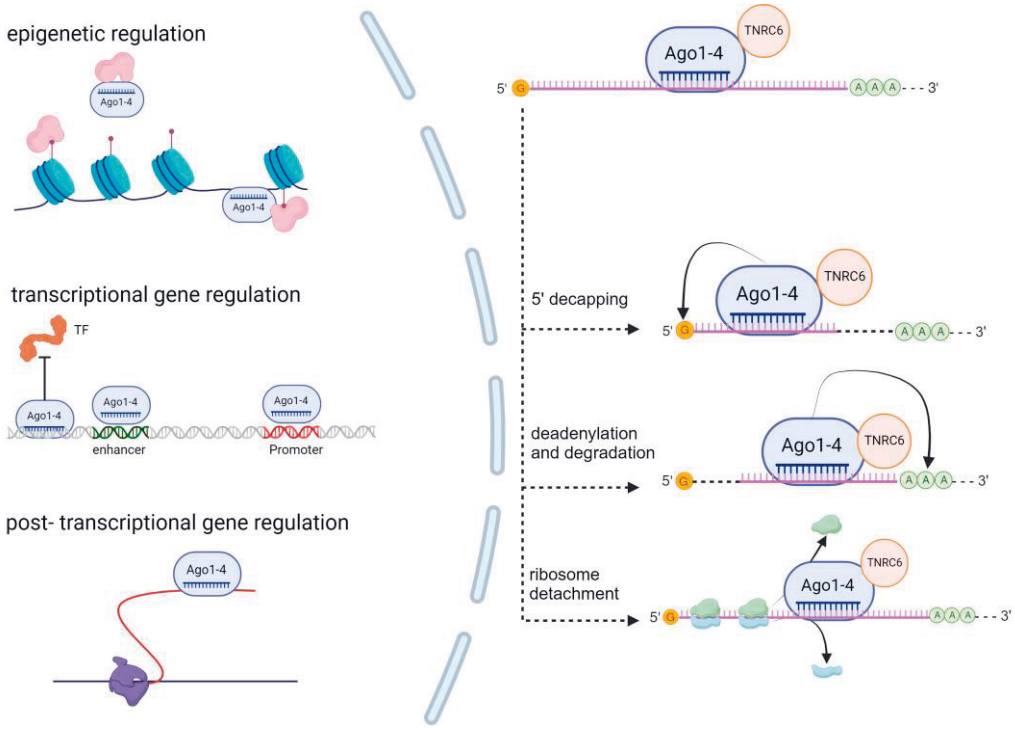

Figure 5: **The roles of miRNAs in the different cellular compartments.** Within the nucleus, miRNAs can act as either transcriptional regulators, influencing gene expression initiation, or post-transcriptional regulators by modulating mRNA stability or processing. In the cytoplasm, miRNAs primarily mediate post-transcriptional gene silencing (PTGS) through three main pathways: mRNA de-capping, de-adenylation and degradation, as well as translational inhibition via ribosome detachment.

2.6.8 Cytoplasmic miRNAs

Once the RISC complex is formed in the cytoplasmic compartment, the miRNA will guide the complex to the target RNA that bares the reverse-

complement of the miRNA nucleotide sequence (Agarwal *et al.*, 2015). The behaviour of the RISC complex is similar to that of a RNA-binding protein as it scans the RNA with a rapid but weak and metastable binding until it becomes long-lived in presence of sequences that is reverse complementarity with the seed sequence of the miRNA (Chandradoss *et al.*, 2015; Salomon *et al.*, 2015; Klum *et al.*, 2018). In the case of a perfect complementarity sequence match between the miRNA and the MREs (miRNA response elements) from the target RNA, the RNA will be cleaved and degraded by the endonuclease activity of AGO2 (Jo *et al.*, 2015). Following this, the association between the 3’ end of the miRNA and the AGO protein will be destabilized, promoting the degradation of the miRNA (Krützfeldt *et al.*, 2005; Ameres *et al.*, 2010). In most cases, MREs contain mismatches with their miRNA preventing AGO2 endonuclease activity and promote translational repression rather than mRNA cleavage and degradation (Jonas and Izaurralde, 2015). In such cases, target mRNA translational repression occurs through de-adenylation and de-capping (Behm-Ansmant *et al.*, 2006; Jackson & Standart, 2007; Wu *et al.*, 2006). RNA de-adenylation is mediated by the recruitment of the poly(A)-de-adenylase complexes PAN2-PAN3 and CCR4-NOT to the RISC complex (Christie *et al.*, 2013; Jonas and Izaurralde, 2015). Later, de-capping occurs through DCP2 (de-capping protein 2) and is followed by the 5'−3' degradation of the mRNA by XRN1 (exoribonuclease 1) (Behm-Ansmant *et al.*, 2006; Braun *et al.*, 2012).

2.6.9 Nuclear miRNAs

Despite being thoroughly investigated for their role in post-transcriptional gene silencing, miRNA and RISC components are also found in the nucleus, indicating that miRNAs are able to regulate genes at the transcriptional level as well (Khraiwesh *et al.*, 2010).

Up until now, most studies have proven that miRNAs bind to the 3’UTR of their target mRNA sequence causing mRNA de-adenylation, de-capping and translational repression (Huntzinger and Izaurralde, 2011; Ipsaro and Joshua-Tor, 2015). However, miRNA can also bind to other regions of mRNA such as the 5’ UTR and the coding sequences, in order to invoke gene

silencing, (Forman *et al.*, 2008; Zhang *et al.*, 2018) or even to promoter regions to induce transcription (Dharap *et al.*, 2013).

One of the numerous mechanisms found to be behind the transcriptional expression control exerted by miRNAs is the interaction between miRNAs and epigenetic regulators. Amongst them is EZH2 (enhancer of zeste homolog 2, also known as KMT6A), the catalytic core of the repressive PRC2 (polycomb repressive complex 2) that acts to increase the level of the repressive H3K27me3 levels on histones, thus inactivating the gene promoter by reducing chromatin accessibility and thereby repressing gene transcription. Interaction between EZH2 and nuclear miRNAs has been observed at different maturation levels. As an example, at the pri-miRNA level, pri-miR-208b binds with EZH2 and mediates the expression level of hypertrophic and fibrotic genes in murine heart tissue (Mathiyalagan *et al.*, 2014). Additionally, EZH2 can interact with mature miRNA. An example of this is miR-320 which leads to the silencing of *POLR3D* gene expression (Kim *et al.*, 2008) or with let-7d, where it is involved in the transcriptional silencing of bidirectionally expressed genes and nucleolar organization (Singh *et al.*, 2018).

In addition, mature miRNAs binding have been observed to bind with MECP2 (methyl-CpG binding protein 2), a protein that specifically recognizes and binds to methylated DNA in order to read the information stored in DNA methylation patterns. For example, miR-375, miR-126, miR-455, miR-542 and miR-let7i, amongst others, have been shown to influence MECP2 interactions with chromatin, thereby regulating gene transcription (Khan *et al.*, 2017).

Indeed, the interaction between miRNAs and epigenetic regulators can occur through the classical miRNA-mediated PTGS pathway, where it can downregulate the expression of DNA methyltransferase genes such as *Dnmt1*, *Dnmt3a*, *Dnmt3b* and *Uhrf1*, thereby decreasing DNA methylation (Farina *et al.*, 2020).

Transcriptional control of nuclear miRNAs also arises from their binding to complementary sequences in target gene promoters or enhancer regions. Regarding miRNA binding to promoter regions, transcriptional activation has been reported with the direct binding of the miRNA to the

TATA-box of specific genes. This association promotes the assembly of the pre-initiation transcription complex by binding RNA polymerase II and the TATA-box binding protein alongside with AGO1, as has been observed with miR-138, miR-92a and let-7i (Huang *et al.*, 2013; Zhang *et al.*, 2014). Conversely, the binding of nuclear miRNA to the TATA-box of the promoter or to the DNA-binding motifs of TFs can prevent their association with their targets and thereby repress gene transcription (Guo *et al.*, 2021).

Concerning nuclear miRNAs binding to enhancer regions, more than 300 miRNA binding sites have been identified within active enhancer regions harbouring H3K27ac, H3K4me1, p300/CBP, or DNase-I-hypersensitivity enrichments sites in one study (Xiao *et al.*, 2017). Indeed, the binding of nuclear miRNAs to enhancer regions was able to activate transcription through chromatin remodelling at enhancer regions, as well as aid the recruitment of p300/CBP and RNA polymerase II (Xiao *et al.*, 2017). Interestingly, the cross-talk between miRNAs and enhancers has been highlighted since super-enhancers have been shown to drastically amplify expression of miRNA by increasing pri-miRNA transcription, facilitate Drosha/DGCR8 recruitment and consequently pri-miRNA processing (Suzuki, Young and Sharp, 2017; Moreau *et al.*, 2021).

Nuclear miRNAs are also able to play a role of nuclear interference. This role is made possible due to the nuclear localization of necessary components such as AGO2 and TNRC6A (Trinucleotide Repeat Containing Adaptor 6A) (Gagnon *et al.*, 2014). Interestingly, the mechanisms of target identification are similar between cytoplasmic and nuclear complexes as they both require pairing between the miRNA seed sequence and its corresponding MRE to trigger the endo-nucleolytic activity of AGO2 (Gagnon *et al.*, 2014; Sarshad *et al.*, 2018). In summary, the nuclear localization of the RNA interference machinery greatly expands the potential target repertoire for miRNA-mediated gene silencing as it includes introns as well as nuclear non-coding RNAs, including lncRNAs and pri-miRNAs (Sarshad *et al.*, 2018; Tang *et al.*, 2012; Dong Wang *et al.*, 2018; Weinmann *et al.*, 2009).

3 AIMS OF THE STUDY

The core objective of this thesis was to characterize the transcriptional and post-transcriptional profiles of atherosclerosis-associated cell types subjected to pro-atherosclerotic stimuli.

More specifically, the studies aimed to achieve the following three objectives:

- Compare the nascent pri-miRNA and mature miRNA profiles of atherosclerosis-associated cell types in response to pro-atherogenic stimuli (Manuscript I)
- Characterize the expression profiles of nascent and steady-state non-coding RNAs in endothelial cells under hypoxic conditions (Manuscript II)
- Investigate the subcellular compartment dynamics of non-coding RNAs in endothelial cells, in response to hypoxia (Manuscript III)

4 MATERIALS AND METHODS

The materials and methods used in this thesis are summarized in the following tables. The detailed descriptions of the methods can be found from the original publications (I to III).

Table 1. Cell lines used in this study.

Cell line	Source	Used in
Human umbilical vein endothelial cell (HUVEC)	Extracted from umbilical cords as described in manuscripts I, II and III Or purchased from LONZA, #CC-2517	I, II, III
Human aortic endothelial cells (HAEC)	LONZA #CC-2535	I
Human aortic smooth muscle cells (HASMC)	LONZA #CC-2571	I
Human monocytes (CD14+) differentiated into Mϕs by MCSF exposure	LONZA #2W-400A	I

Table 2. Stimulations used in this study.

Stimulus	Description	Source	Used in
Human recombinant Macrophage Colony Stimulating Factor (rHu M-CSF)	Cytokine that controls differentiation, and function of monocytes into Mϕs	ThermoFisher Scientific	I
Hypoxia	Mimics the signal that induces the thickening of the aortic vessel wall	Baker Ruskinn	I, II, III

1-palmitoyl-2-archidonoyl-sn-glycero-3-phosphocholine (oxPAPC)	Mimics an intermediate atherosclerotic state where cells are in contact with oxidized lipids	Avanti Polar Lipids Inc	I

Table 3. Kits and other reagents used in this study.

Kit / reagent	Description and usage	source	Used in
RNeasy Mini Kit	RNA isolation from cultured cell For RT-qPCR and RNA-Seq	QIAGEN	I
FastStart™ Universal SYBR® Green Master (Rox)	Hot start reaction mix for qPCR and RT-qPCR	Merck	I
ExiLENT SYBR® Green master mix	SYBR green based method for miRNA PCR amplification and detection though RT-qPCR	Exiqon	I, III
miRCURY LNA miRNA PCR assay primer mix has-miR-100-5p and SNORD48	Detection and quantification of miRNA	Exiqon QIAGEN	I
SYBR Green ER master mix	Reaction mix used for the amplification and detection of DNA or cDNA frangment for qPCR and RT-qPCR	Invitrogen	I
CyQUANT™ NF Cell Proliferation Assay	Measurement of cell proliferation via fluorescent dye binding on cellular DNA	ThermoFisher scientific	III
Lipofectamine 3000	ASOs and miRNAs mimics transfections	Thermo Fisher Scientific	I, III
RNase-free DNase set	DNA digestion prior to cDNA synthesis	Qiagen	I, III

DNase I	DNA digestion in RNA samples prior to cDNA synthesis	Thermo Fisher Scientific	I, III
RevertAid first-strand cDNA synthesis kit	cDNA synthesis for RT-qPCR	Thermo Fisher Scientific	I, II
Ribonuclease H	Degradation of RNA after cDNA synthesis	Thermo Fisher Scientific	I, II
RNA Kit for Fragment Analyzer	Quantitative and qualitative analysis of total RNA by capillary electrophoresis for RNA-Seq	Agilent Technologies	I, III
Qubit dsDNA HS assay kit	RNA-Seq and GRO-Seq libraries quantification	Thermo Fisher Scientific	I, III
SUPERase Inhibitor	RNAses inhibitors used for GRO-Seq and RNA-Seq	Thermo Fisher Scientific	I, II, III
RNA fragmentation reagent	fragment RNA to sizes between 60–200 nucleotides for GRO-Seq and RNA-Seq	Thermo Fisher Scientific	I, II, II
TRIzol LS Reagent	RNA isolation	Thermo Fisher Scientific	II, III
Poly(A) polymerase	Poly(A) tailing of RNA used for GRO-Seq	New England Biolabs	I, II
Superscript III Reverse Transcriptase	Reverse transcriptase used for cDNA synthesis in GRO-Seq	Thermo Fisher Scientific	I, II
Exonuclease I	Removal of nucleotides from single-stranded DNA used in GRO-Seq	New England Biolabs	I, II
ChIP DNA Clean & Concentrator Kit	DNA purification and concentration for GRO-Seq	Zymo Research	I, II

CircLigase ssDNA Ligase	Catalyzes the circularization of ssDNA templates for GRO-Seq	Epicentre	I, II
10% NOVEX TBE gel	Polyacrylamide gels allowing separation of double-strand DNA fragments for library size selection used in GRO-Seq	Thermo Fisher Scientific	I, II
TURBO DNase	Cleaves double-stranded DNA to clear DNA contamination used in RNA-Seq	Thermo Fisher Scientific	I, II
RNA Clean & Concentrator	RNA cleaning kit used for RNA-Seq and miR-Seq	Zymo Research	I, II
Ribo-Zero Gold rRNA Removal Kit	Removes rRNA from total RNA used for RNA-Seq	Illumina	I, II
Oligofectamine	Mimic and inhibitor miRNA transfections	Invitrogen	I
QuantSeq-Pool Sample-Barcoded 3' mRNA-Seq Library Prep Kit for Illumina	RNA-Seq library preparation,	Immuno Diagnostics	III
CyQuant™ NF Cell Proliferation Assay	Measurement of cell proliferation	Thermo Fisher	III

Table 4. Bioinformatics tools used in these studies.

Package	Description	Used in
HOMER	Suite of tools for Motif Discovery and next-generation sequencing analysis	I, II, III
Bowtie	Aligner of short DNA sequences to the human genome used for ChIP-seq and GRO-seq experiments	I, II, III
edgeR	Differential expression analysis of RNA-seq	I, II, III
FastQC	Quality control tool for high throughput sequencing	I, II, III

STAR	RNA-seq alignment algorithm from RNA-Seq and miRNA-Seq experiments	I, II, III
UCSC Genome Browser	Human genome assembly used to visualize NGS data	I, II, III
MiRbase database	Database of published miRNA sequences and annotation	I
NF-core RNAseq	Bioinformatics pipeline used to analyse RNA sequencing data	I, III
Trim Galore!	Quality and adapter trimming as well as quality control of sequencing reads	I, II, III
CiiiDER tool	Predict and analyze transcription factor binding sites	I
custom de novo pri-miRNA detection pipeline	Identification of pri-miRNAs transcripts variants DOI: 10.1093/nar/gkx909	I
DESEQ2	Differential gene expression analysis based on the negative binomial distribution	I, III
ComplexHeatmap	Visualize associations between different sequencing experiments	I, III
fGSEA	Algorithm for fast gene set enrichment analysis	I, III
Ingenuity Pathway Analysis	Analysis of gene expression patterns and networks using a build-in scientific literature-based database	I, II, III
miRWalk	Identification of miRNA target genes	I, III
IPA (Ingenuity Pathway Analysis; Qiagen)	Identification of canonical pathways and upstream regulators	I, II, III
BEDTools	Set of tools used for NGS data analysis tasks	I, II, III
Idemux	Demultiplexing paired-end FASTQ files	III

bcl2fastq2 Conversion Software (v2.20)	Demultiplexing sequencing data and converting base call (BCL) files into FASTQ files.	III

Table 5. Oligonucleotides used in these studies.

Target	ID or sequence (5' to 3')	Used for (in)
MISSION miRNA Mimics Negative Control #1	Sigma-Aldrich #HMC0002	miRNA overexpression (I)
hsa-miR-100-5p mimic	Sigma-Aldrich #HMI0023	miRNA overexpression (I)
MISSION Synthetic miRNA Inhibitors Negative control I	Sigma-Aldrich #ncstud001	miRNA silencing (I)
hsa-miR-100-5p inhibitor	Sigma-Aldrich #HSTUD0023	miRNA silencing (I)
SNORD48	Exiqon #203903	RT-qPCR (I)
BMPR2	Forward: AACAACAGCAATCCATGTTC Reverse:TATCTGTATACTGCTGCCATC	RT-qPCR (I)
SMAD7	Forward:CAGATTCCCAACTTCTTCTG Reverse:CTCTTGTTGTCCGAATTGAG	RT-qPCR (I)
LATS2	Forward:TGGATGGTCACATTAAACTC Reverse:CTCCCTTTCTGGTAATATTTGG	RT-qPCR (I)
PPP2R2D	Forward:ACAAAATCAACTAGAGACGC Reverse:ACAATGATGCCTTTTCTCTG	RT-qPCR (I)
PRKCZ	Forward:AGAGGACTACCTTTTCCAAG Reverse:TTAAAACATGGGAGGCTTTG	RT-qPCR (I)
AJUBA	Forward:GAAGATCCTACAAGCAATGG Reverse:TATACTTGGTTGGAGAAGTCC	RT-qPCR (I)
ATP5F1	Forward:GCCCTGACAGATTCTCCTATCG Reverse:GAAAGGTCCTTGTTGCCTGC	RT-qPCR (I)

RPLP0	Forward:GGAGACGGATTACACCTTCCC Reverse:CAGCCACAAAGGCAGATGG	RT-qPCR (I)
LUCAT1	Qiagen Antisense LNA GapmeRs LUCAT1 : LG00780246-DDA	RNAse-H mediated Knockdown (III)
SZT2-AS1	Qiagen Antisense LNA GapmeRs SZT2-AS1_1 : LG00780255-DDA	RNAse-H mediated Knockdown (III)
SLC2A1-AS1	Qiagen Antisense LNA GapmeRs SLC2A1-AS1_1 : LG00778843-DDA SLC2A1-AS1_2 : LG00778844-DDA	RNAse-H mediated Knockdown (III)
A2M-AS1	Qiagen Antisense LNA GapmeRs A2M-AS1_2 : LG00778879-DDA A2M-AS1_6 : LG00778883-DDA	RNAse-H mediated Knockdown (III)
SNHG15	Qiagen Antisense LNA GapmeRs SNHG15_3 : LG00778890-DDA SNHG15_9 : LG00778896-DDA	RNAse-H mediated Knockdown (III)
SDCBP2-AS1	Qiagen Antisense LNA GapmeRs SDCBP2-AS1_1 : LG00778898-DDA SDCBP2-AS1_9 : LG00778906-DDA	RNAse-H mediated Knockdown (III)
Negative Control	Qiagen Antisense LNA GapmeRs LG00000002-DDA	RNAse-H mediated Knockdown (III)

Table 6. Antibodies used in these studies.

Target antigen	Product information	Used for (in)
pTAZ (Ser89)	Cell Signaling Technology, #59971	Western blot (I)
YAP/TAZ	Cell Signaling Technology, #8418	Western blot (I)

Table 7. Sequencing and laboratory methods used in these studies

Method	Description	Used in
Global run-on sequencing (GRO-Seq)	Sequencing of nascent RNA transcriptional pool	I, II

RNA sequencing (RNA-seq)	Sequencing of mature mRNA expression pools	I, II, III
miRNA sequencing (miRNA-seq)	Sequencing of mature miRNA expression pools	I
Chromatin immunoprecipitation followed by sequencing (ChIP-seq)	Identification of DNA regions containing specific histone modifications or DNA-binding proteins	I, II
Quantitative reverse transcription PCR (RT-qPCR)	RNA expression analysis	I, III
Proliferation assay	Quantification of cell proliferation	I, III
Cell fractionation	Separation of cell nucleus and cytoplasm (Gagnon *et al.*, 2014)	III

Table 8. Data availability.

Description	Source / Repository	Dataset description / URL
GSE136813	NCBI Gene Expression Omnibus	GRO-Seq, RNA-Seq and miRNA-Seq of HAEC, HUVEC, HASMC, CD14+/ Mϕs analysed under hypoxia, oxPAPC and hypoxia+oxPAPC https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE136813
GSE154427	NCBI Gene Expression Omnibus	GRO-Seq, RNA-Seq and miRNA-Seq of HAEC, HUVEC, HASMC, CD14+/ Mϕs analysed under hypoxia, oxPAPC and hypoxia+oxPAPC https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE154427
GSE44288	NCBI Gene Expression Omnibus	ChIP-Seq and RNA-seq of Med1, Oct4 and Sox2 in mouse pluripotent embryonic stem cells. https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE44288

GSE52642	NCBI Gene Expression Omnibus	ChIP-Seq (H3K4me2), GRO-Seq and HiC profiling was performed in HUVECs and HAECs. https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE52642
GSE26497	NCBI Gene Expression Omnibus	miRNA-seq of HUVEC under normoxia and 24h of hypoxia https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE26497
GSE51522	NCBI Gene Expression Omnibus	ChIP-Seq (H3K27ac) in Jurkat T-ALL cell line https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE51522

5 RESULTS AND DISCUSSION

5.1 PRIMARY AND MATURE MIRNA EXPRESSION CHANGES UPON EXPOSURE TO PRO-ATHEROGENIC STIMULI

5.1.1 Pri-miRNA promoter usage and transcript variant expression in atherosclerosis-associated cell types

A sizable part of the human genome encodes ncRNAs and, amongst them, miRNAs originate from primary transcripts that undergo extensive processing to produce singular or multiple mature miRNAs, as described in the introduction to this thesis.

To study the nascent pri-miRNA expression profile differences between the different atherosclerosis-associated cell types, we generated GRO-Seq data for HAECs, HUVECs, HASMCs, and CD14+/MΦs. Using a custom *de-novo* detection pipeline based on GRO-Seq, as well as matching ChIP- and CAGE-Seq data originating from 27 various human cell types (Bouvy-Liivrand *et al.*, 2017), we identified 781 pri-miRNA transcripts expressed in at least one condition in all replicates of a single cell type. Among these pri-miRNAs, 87% were differentially expressed between the cell types under all studied conditions and for 453 the difference was over 10-fold in at least one pair-wise comparison of cell types **(Manuscript I – Figures 1A and 1B)**. Of these, 84 pri-miRNAs were only expressed in a specific cell-type manner confirming the strong cell-type specificity of pri-miRNA transcript variants (TVs) **(Manuscript I – Figure 1E)**. Interestingly, we could neither highlight stimuli-specific patterns in TSS usage nor TV transcription, suggesting that the pri-miRNA expression is regulated in a cell-type specific manner, most likely through the usage of cell-type specific enhancers that largely pre-exist before exposure to stimulation (Heinz *et al.*, 2015) **(Manuscript I – Figure 1C)**.

5.1.2 Differential expression changes of pri-miRNA pools in response to single or combined treatments

Next, we looked deeper into the pri-miRNA expression profiles upon exposure to pro-atherogenic stimuli. After stimulation of cultures of the 4 cell lines with hypoxia, oxPAPC and hypoxia+oxPAPC, we identified 569, 430, and 515 differentially expressed pri-miRNAs (GRO-seq, FDR <0.05), respectively. Interestingly, most pri-miRNAs showed the same direction of expression under all stimuli, with only 11% displaying stimuli selective specificity in their expression **(Manuscript I – Figure 3A and 3C)**. These results showed that hypoxia, oxPAPC, and combined treatment have the same directional effect on pri-miRNA regulation in the studied cell type which implies that similar transcriptional regulatory mechanisms could be involved in the regulation of pri-miRNAs, upon exposure to these two pro-atherogenic stimuli.

To investigate further the commonalities in the transcriptional mechanisms, we examined the TSS of deregulated transcripts (fold change >2 in at least one comparison, in at least one cell type) for the presence of *de novo* DNA motif enrichment, upon stimulation. Altogether, we detected 679, 140, and 172 motifs enriched across the genome in hypoxia, oxPAPC and hypoxia+oxPAPC, respectively and 1, 329 and 361 motifs depleted for hypoxia, oxPAPC, and hypoxia+oxPAPC, respectively, in comparison to the control cell samples **(Manuscript I – Figure 3D)**. When exposed only to oxPAPC stimulus, cells displayed 1 motif (TGIF1) that was specifically enriched and not shared with any other condition, while a larger set of stimulus-specific motifs were over-represented upon exposure to hypoxia. Interestingly, only 2 motifs were specific to hypoxia+oxPAPC stimuli (THAP1 and TBX3), confirming that most enriched motifs were shared upon stimulation with a singular stimulus. Indeed, in hypoxia+oxPAPC condition, we detected signal responsive TFs such as ARNT (aryl hydrocarbon receptor nuclear translocator), also known as HIF1-β (hypoxia-inducible factor 1, beta subunit) controlling the hypoxia response by binding to HIF1α (Mandl and Depping, 2014). Furthermore, ARNT has been recognized as a mediator of the oxidative stress response through the activation of NRF2 (nuclear factor

erythroid 2-related factor 2) (Ma *et al.*, 2004), which is in line with the recently discovered crosstalk between NRF2 and HIF1α signalling pathways (Ji *et al.*, 2018; Johansson *et al.*, 2017; Lacher *et al.*, 2018). Amongst the top enriched TFs binding motifs, we also identified Zfp148 (zinc-finger protein 148) that has been shown to exhibit pro-atherogenic effects *in vivo* by promoting cell proliferation under oxidative stress (Sayin *et al.*, 2014) and EGR-1 (early growth response 1) which has been associated with atherosclerosis progression (Furnkranz *et al.*, 2005). Our analysis also identified various members of the KLF (Krüppel-like factor) family of TFs, such as KLF2, 4, 6, and 11, which have been shown to contribute to pro-atherogenic gene expression changes and to participate in various biological functions, such as the regulation of inflammation, angiogenesis, and thrombosis in both endothelial and smooth muscle cells. (Fan *et al.*, 2017; Raitoharju *et al.*, 2013).

In conclusion, the analysis of pri-miRNA transcription profiles across different cell types and under various stimuli reveals a remarkable cell-type specificity in pri-miRNA expression and a surprising similarity in the response to different proatherogenic stimuli, suggesting that common regulatory mechanisms exist that involve cell-type specific enhancers and shared transcription factors.

5.1.3 Mature miRNAs expression differences between cell types and stimuli and its influence on target genes

Next, we examined the mature miRNA profiles of HUVECs, HAECs, HASMCs, and CD14+/Mϕs, in response to pro-atherogenic stimuli. Using miRNA-Seq data, we were able to establish a list of about 500 miRNAs that were found to be expressed in at least one of the different cell types **(Manuscript I – Figure 2A)**. To our surprise, only a small fraction (18%) of the mature miRNAs were defined as cell-type specific **(Manuscript I – Figure 2B and Supplementary Figures IIIA and IIIB)**. Curiously, only 40% of these cell-type specific mature miRNAs were cell-type specific at the level of nascent transcription suggesting that the processing and turnover of miRNAs is the major determinant driving cell-type specific expression of mature miRNAs **(Manuscript I – Supplementary Figure IVA)**. For instance,

the efficiency of the microprocessor complex to convert pri-miRNAs into pre-miRNA could be governed by the sequence of the pri-miRNA itself, alongside secondary structural features, as well as differential microprocessor cofactors (Conrad *et al.*, 2014). Moreover, other non-coding RNAs and proteins can bind the microprocessor complex, or the pri-miRNA itself, to participate in the post-transcriptional regulation of miRNAs (Creugny, Fender and Pfeffer, 2018). In addition, mature miRNAs levels are determined by multiple factors. Firstly, the availability of the proteins necessary for their biogenesis and stability, such as DICER and AGO, in addition to secondary structural features of the pri-miRNA, as well as their subcellular localization, nuclear import/export and cytoplasmic processing (Weinmann *et al.*, 2009; Gibbings *et al.*, 2012; Conrad *et al.*, 2014; Pitchiaya *et al.*, 2017; Santovito *et al.*, 2020). In addition, the decay rate of miRNAs constitutes another mechanism by which the levels of miRNA are controlled. The degradation dynamics of miRNAs is achieved through enzymatic activities which are able to modify the miRNA ends, either by adding ("tailing") or removing ("trimming") nucleotides and through the action of target-dependant mechanisms of miRNAs with their mRNA targets, leading to AGO unloading and degradation (la Mata *et al.*, 2015; Lee *et al.*, 2014; Ramachandran & Chen, 2008; Rüegger & Großhans, 2012). The combination of all these post-transcriptional mechanisms regulating the levels of miRNA and the fact that many pri-miRNA fail to generate miRNAs (Bedi *et al.*, 2020) could explain the low correlation observed between the expression of pri-miRNAs and their corresponding mature miRNAs.

There have been many studies examining miRNA expression during atherosclerosis, however, most of them analyzed their deregulation in bulk tissue samples that are composed of many cell types. Consequently, little is known on the cells of origin of the differential signals, and whether they are intra-cellular or inter-cellular in nature. To address this particular knowledge gap, we gathered information on the differentially expressed miRNAs in human atherosclerotic plaques from 5 published studies (Cipollone *et al.*, 2011; Raitoharju *et al.*, 2011; Maitrias *et al.*, 2015; Markus *et al.*, 2016; Parahuleva *et al.*, 2018) and compared their expression level in each of the

three different cell types (ECs, HASMCs, and CD14+/Mϕs) under the different stimuli conditions.

Interestingly, we observed a highly cell-type specific expression pattern of the atherosclerotic plaques associated miRNAs amongst the three cell types (ECs, HASMCs, and CD14+/Mϕs), and for 85% of them, their corresponding pri-miRNAs were found to be differentially expressed (FDR <0.05) in at least one condition **(Manuscript I – Figure 4A)**. This implies that disease-relevant miRNA dysregulation is determined at the transcriptional level and its origin of expression can be pinpointed to one particular cell type. Moreover, even though the mature miRNA profiles of each studied cell type may not show substantial differences between cell types or under proatherogenic stimuli, the functions of miRNAs can vary between them due to differences in their target gene pools. Here, we reported that around 5% of mRNA molecules are exclusively expressed in one cell type or exhibit a more than 8-fold difference in expression levels between cell types. Additionally, this potential target gene pool of miRNAs is more prone to be modulated by different stimuli as well **(Manuscript I – Supplementary Figures XA and XB)**.

Remarkably, The Functional Annotation of the Mammalian Genome (FANTOM) project has shown that miRNA expression levels vary greatly between cell types and is highly skewed since, on average, only 5 miRNAs contributed to half of the total miRNA expression (De Rie *et al.*, 2017). Similarly, here, we found that 2 to 5 species of miRNAs in the studied cells represented more than half of the total miRNA pool expressed within the respective cell types **(Manuscript I - Figure 2E)**. In addition, the 10 most abundant miRNAs species account for 70 to 90% of the miRNA pools **(Manuscript I - Figure 2F)**. Noteworthily, we observed that the top 10 miRNAs were located closer to super-enhancers, offering a potential mechanism explaining their high expression level **(Manuscript I - Figure 2G)**. Substantiating this claim, it has been suggested that super-enhancers play an important role in the production of cell-type specific miRNAs. Specifically, these super-enhancers promote the transcription of nascent pri-miRNAs by facilitating the recruitment of the Drosha/DGCR8 complex, ultimately boosting miRNA production (Suzuki, Young and Sharp, 2017).

Looking at the mRNA targets of the 5 most abundant miRNAs (miR-21-5p, miR-100-5p, miR-22-5p, miR-34a-5p, and miR-92a-3p) expressed in the studied cell types, we found that the HIPPO pathway is one of the main targets of these most highly expressed miRNAs **(Manuscript I – Figure 4D)**. Briefly, when the HIPPO pathway is activated, the YAP/TAZ complex is unphosphorylated and it is translocated into the nucleus in order to exert its effects on gene regulation. Conversely, when the HIPPO pathway is repressed, the YAP/TAZ remains phosphorylated and is restricted to the cytoplasm. When the HIPPO pathway and, consequently the YAP/TAZ complex is activated, it can either induce or repress inflammation, angiogenesis and proliferation, depending on the cellular context (Grundmann *et al.*, 2011; Ben Mimoun and Mauviel, 2018; Oceandy *et al.*, 2019). In our analysis of the predicted miRNA targets, the HIPPO pathway was predicted to be repressed and the effect was strongest in ECs. Interestingly, the activation of YAP/TAZ in ECs has been previously implicated in the onset and progression of atherosclerosis (K. C. Wang *et al.*, 2016). Furthermore, among the most expressed miRNAs, induction miR-100-5p has been closely associated with vulnerable plaque phenotypes (Maitrias *et al.*, 2015; Pankratz *et al.*, 2018; Wang *et al.*, 2015).

5.1.4 The role of miR-100-5p in atherogenesis

To confirm the prediction of functional effects of miRNAs, we selected miR-100-5p and modulated its levels, in both HUVEC and HASMC. Subsequently, we determined the cellular localization of phosphorylated and unphosphorylated TAZ, quantified the expression changes of genes within the HIPPO pathway and identified the biological pathways affected by the overexpression of miR-100-5p. Our results show that overexpression of miR-100-5p tended to increase nuclear TAZ levels in pro-atherogenic conditions whilst its silencing reduced TAZ levels in the nucleus. Interestingly, the opposite was observed in HASMC since overexpression of miR-100-5p decreased nuclear TAZ and increased phosphorylated TAZ levels **(Manuscript I – Figure 5A)**. Moreover, the analysis of expression of mRNA targets of miR-100-5p within the HIPPO pathway confirms the cell-type

specificity of miR-100-5p, with the specific regulation of *LATS2* (Large Tumor Suppressor Kinase 2) (in HUVEC), and both *AJUBA* (Ajuba LIM Protein) and *SMAD7* (mothers against decapentaplegic homolog 7) (in HASMC) **(Manuscript I – Figure 5B)**. Noteworthily, the impact of miR-100-5p on *LATS2* and *AJUBA* expression was in the opposite direction to what would be predicted from the canonical miRNA regulation, under the combined stimuli, suggesting a likely indirect effect. In summary, our data demonstrates that the regulation of the HIPPO pathway by miR-100-5p is highly specific to both the cell type and the stimuli used.

We used total RNA-seq profiling of HUVEC and HASMC transfected with a miR-100-5p mimic, subjected to the combined stimuli of oxPAPC and hypoxia to provide more information on the nature of the cell type specific responses and biological pathways affected by miR-100-5p **(Figure 6)**. We observed a clear cell-type specific effect with the hallmark gene sets related to hypoxia, TNFα (tumour necrosis factor-alpha) signalling via NF-κB (nuclear factor kappa B subunit 1), and IFNγ (interferon gamma) response pathways negatively enriched in HUVECs and positively enriched in HASMCs. On the other hand, we observed a significantly (FDR <0.1) positive enrichment for genes related to proliferation (Mitotic spindle) and negative enrichment for mTORC (mechanistic target of rapamycin kinase) signalling and ROS (reactive oxygen species) pathways, in HUVECs only. At the same time, in HASMCs, genes associated with TGF-β (transforming growth factor beta 1) signalling and apoptosis were positively enriched **(Manuscript I – Figure 5C)**. Interestingly, *SMAD7*, which is negatively regulated by miR-100-5p solely in HASMCs, under proatherogenic stimuli, has been shown to coordinate the crosstalk between HIPPO- and TGF-β signalling (Nakao, Okumura and Ogawa, 2002; Qin *et al.*, 2018) where it suppresses the TGF-β signalling pathway, providing a potential mechanism for explaining the cell-type specificity of TGF-β pathway hallmark genes enrichment in HASMCs that we found.

Interestingly, a recent study has highlighted the cross-talk between mTORC and HIPPO pathways by concluding that when one pathway is on the other is turned off (Nakao, Okumura and Ogawa, 2002). Additionally, it has been observed that the repression of mTORC signalling is associated

with an anti-inflammatory reaction to atherosclerotic stimuli in HUVECs, under the condition of miR-100-5p overexpression (Pankratz *et al.*, 2018). Furthermore, our findings provide a potential justification for the varying functional consequences of miR-100 in a disease context as its inhibition leads to significantly increased migration of HASMCs but not in HUVECs (Grundmann *et al.*, 2011).

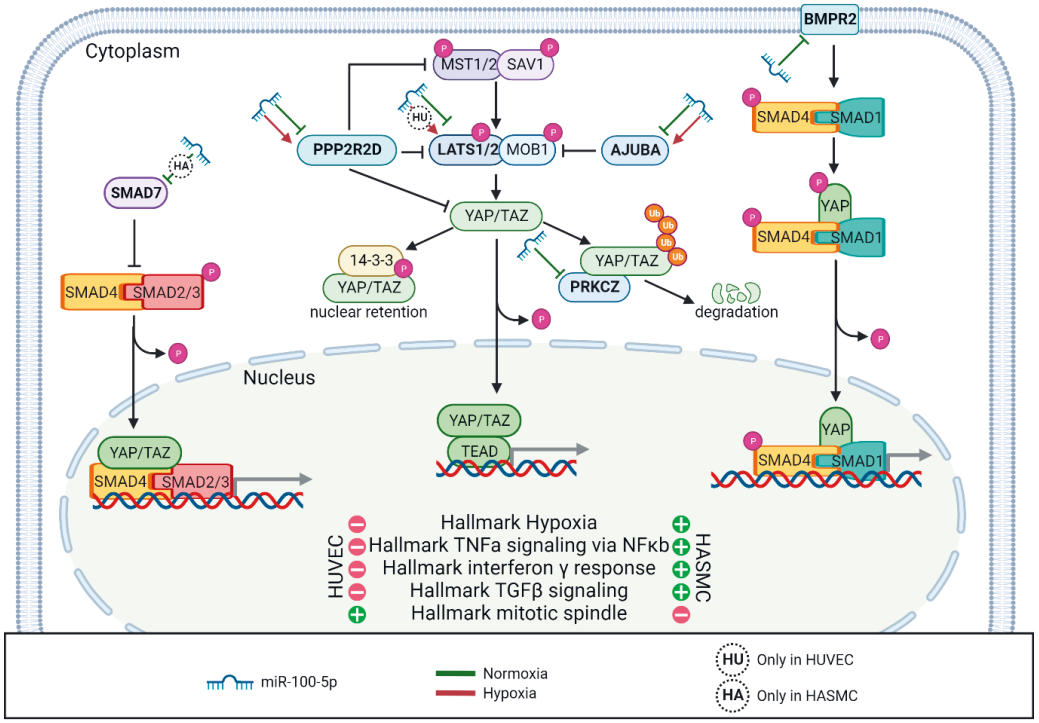

Figure 6: **Schematic of the factors implicated in the HIPPO pathway that were investigated in Manuscript I.** Interactions between miR-100-5p and key factors implicated in the HIPPO pathway influence YAP/TAZ activity, a critical regulatory node in the HIPPO pathway. Gene names in bold represent the targets of miR-100-5p.

Altogether, this study revealed that transcriptional and post-transcriptional regulation of miRNA is more complex than previously understood and provides a resource for exploring the variations of cell-type specific miRNAs transcription in the vasculature. Moreover, we clearly pinpoint miR-100-5p as a modulator of the cell-type specific regulation of inflammation and hypoxia associated genes. Ultimately, these findings have

the potential to advance our knowledge of the regulatory networks that are relevant in atherosclerosis and could lay the groundwork for the development of cell-targeted therapeutics (Bader, 2012; Janssen *et al.*, 2013).

5.2 TRANSCRIPTIONAL PROFILING OF LONG NON-CODING RNAS UPON HYPOXIA

5.2.1 Hypoxia regulation of the coding and non-coding transcriptome

Long ncRNAs represent a major part of the transcriptome but their expression profiles and regulation are poorly studied. This is partly due to low stability of lncRNAs and an inefficiency in their capture using polyA-based RNA-Seq. To address this gap in knowledge, we used GRO-Seq to measure the nascent transcription. Using GRO-seq not only circumvents the issues associated with the low stability of lncRNAs but also enhances efficiency in capturing their dynamics, providing a more robust and reliable assessment of their transcriptional activity (Kaikkonen *et al.*, 2013).

Hypoxia occurs during development but it also represents one of the hallmarks of ischemic cardiovascular diseases, affecting cells of the arterial wall (Wong *et al.*, 2017). In addition, ECs exert a critical role in atherosclerosis. In the early phase, endothelial injury and the resulting dysfunction stimulates leukocyte infiltration into the vessel wall (Nabel and Braunwald, 2012). In the latter stages, ECs are exposed to an adverse microenvironments, containing conditions such as hypoxia and proinflammatory stimuli, that could lead to the disruption of the endothelial barrier (Wong *et al.*, 2017). Moreover, ECs exposed to hypoxia have been seen to respond through the activation of cell proliferation, migration and angiogenesis aiming to repair and resolve the hypoxic conditions.(Wong *et al.*, 2017). However, the diversity, expression dynamics and functions of lncRNAs in ECs is still poorly understood. Therefore, this study aimed at shedding light on ECs response to hypoxia through lncRNA expression dynamics. Furthermore, by profiling lncRNAs under hypoxic conditions in ECs, we sought to identify novel lncRNA-mediated regulatory pathways that could contribute to cardiovascular disease development.

Analysis of GRO-Seq results allowed us to identify 808 known lncRNAs based on NONCODE v5 database annotations (Fang *et al.*, 2018) and 955 novel transcripts that were differentially expressed under hypoxic conditions. Interestingly, among these transcripts, 544 were upregulated and 350 were downregulated more than 2-fold **(Manuscript II – Figure 1B)**. Amongst the most highly upregulated transcripts, we identified many novel lncRNAs, but also some well-known lncRNAs previously implicated in a pathological context or adverse conditions. Amidst the most upregulated transcripts, the lung cancer-related transcript 1 (*LUCAT1*; also called *SCAL1*), an anti-apoptosis associated lncRNA previously found to affect the proliferation, migration, and invasion of various cancer cells was identified (Xing *et al.*, 2021). *LUCAT1* has been also found to influence DNA methylation levels by regulating *DNMT1*, and *RASSF1-AS1*, a negative regulator of the pro-apoptotic regulator/tumor suppressor RASSF1 (Beckedorff *et al.*, 2013; Yoon *et al.*, 2018). In addition, *IDH1-AS1* which modulates IDH1, and consequently the crosstalk between the transcription factors c-Myc and HIF1α that regulates the glucose uptake and control of energy metabolism (Xiang *et al.*, 2018) was identified. In summary, these lncRNAs represent candidates that could increase cell survival and proliferation to promote vascular reorganization upon hypoxia and warrant further study.

5.2.2 Promoter- and enhancer-associated lncRNAs regulation and its impact on nearby protein-coding genes

Next, the lncRNAs were separated into two categories (promoter– or enhancer-associated lncRNAs) based on their enrichment in H3K4me3 and H3K4me1 histone marks. The lncRNAs were considered to be promoter-associated lncRNAs (p-lncRNAs) if they displayed a higher enrichment for H3K4me3 or enhancer-associated lncRNAs (e-lncRNAs) if they showed a higher enrichment for H3K4me1 histone marks (Heintzman *et al.*, 2007). Altogether, 707 lncRNAs were assigned to be p-lncRNAs, whilst 1056 were considered to be e-lncRNAs **(Manuscript II – Figure 2A)**. This distribution is in line with cap analysis of gene expression (CAGE), which concluded that 68% of lncRNAs originate from enhancers (Hon *et al.*, 2017). However, the

definition of enhancers and promoters based on the H3K4me1/H3K4me3 ratio may omit a small portion of functional enhancers but it useful considering that functional characterization has not been achieved yet and the terminologies remain currently ambiguous (Pekowska *et al.*, 2011). Of note, upon exposure to hypoxia, p-lncRNAs were showing an expression pattern that was comparable to that of protein encoding genes with an upregulation of nearly 90% of the transcripts. Conversely, e-lncRNAs showed similar levels of either transcriptional upregulation or downregulation **(Manuscript II – Figure 2A)**.

To determine the potential mechanisms responsible for the differences observed between the regulation of p-lncRNAs and e-lncRNAs, we used *de novo* TF motif analysis to measure DNA-binding motif enrichment +/- 500bp from their transcription start sites. Interestingly, the motif analysis results revealed clear differences in the most highly enriched transcription factors between the two classes of lncRNAs. In the vicinity of e-lncRNAs, the most enriched transcription factors were AP-1/JUN and NKX3, both of which have been previously associated with the control of vascularization under hypoxic conditions (Fantozzi *et al.*, 2003; Im and Kim, 2017). Additionally, MAFA, a regulator of energy homeostasis that can be either be an activator or repressor of transcription (Tsuchiya, 2015) was detected as one of the strongest enriched transcription factor motifs **(Manuscript II – Figure 2B)**. Regarding p-lncRNAs, as expected, the most enriched motifs were promoter-proximal transcription factors binding motifs such as Sp1 and NRF1 (Andersson *et al.*, 2014; Grossman *et al.*, 2018) **(Manuscript II – Figure 2B)**. These transcription factors have been shown to modulate the activity of genes associated with cell growth and proliferation (Black *et al.*, 2001; Duronio & Xiong, 2013; Wang *et al.*, 2006) by binding to G-rich elements such as the GC-box and GT motif (Fulciniti *et al.*, 2011; Xu *et al.*, 2012).

In general, lncRNAs are considered as proximal regulators of gene expression due to the correlation between lncRNA expression and the expression of proximal coding genes (Cabili *et al.*, 2011). Therefore, we correlated the expression changes of the two classes of differentially expressed lncRNAs with nearby protein coding genes exhibiting differential gene expression (FDR value below 5% and a fold change threshold of ±1.5).

Interestingly, for both categories of lncRNAs, the correlation is high between their expression and their proximal protein encoding genes with a stronger correlation for e-lncRNAs than for p-lncRNAs (rho =0.45 and 0.24, respectively) **(Manuscript II – Figure 4A)**. This difference could be explained by the fact that lncRNAs expressed by enhancers are actively involved in transcriptional process (Hah *et al.*, 2011), and are known to regulate proximal coding genes, and their level of expression depends on their activity (Andersson *et al.*, 2014; F. Lai *et al.*, 2015; Lam *et al.*, 2013; Li *et al.*, 2013). In contrast, p-lncRNAs have been seen previously to be able to upregulate or downregulate gene expression and in some cases show negative correlation with the adjacent gene's expression (Lloret-Llinares *et al.*, 2016).

To identify the subgroup of lncRNAs that could affect gene expression in *trans*, we correlated the GRO-Seq expression levels to the RNA-Seq of matching HUVEC donors exposed to the same stimulus. Whilst GRO-Seq reflects nascent transcripts irrespective of their half-lives, RNA-Seq, on the other hand, displays only the steady state lncRNA levels that reflect the situation of both newly synthesized and actively degraded pools of molecules containing the sequences. Therefore, this correlation analysis allows to highlight the abundant and long-lived lncRNAs that could have an impact on gene expression in *trans* (Rinn *et al.*, 2007; Daxinger and Whitelaw, 2012; Kopp and Mendell, 2018). Interestingly, in comparison with protein coding transcripts where 84% of transcripts were detected in both GRO-seq and RNA-seq, only 13% of the lncRNA transcripts were detected in GRO-Seq were also identified in RNA-Seq. These results highlights the variability of the non-coding transcriptome that contains many unstable transcripts (Houseley and Tollervey, 2009). Among the more stable, long-lived, lncRNA transcripts, 76.3% (180/236) was associated with p-lncRNAs, while only 23.7% (56/236) was affiliated to e-lncRNAs. Surprisingly, among the stable transcripts detected in both GRO-Seq and RNA-Seq, e-lncRNAs showed a higher correlation (rho=0.72) than the p-lncRNAs (rho=0.37) **(Manuscript II – Figure 4C and Supplementary Figure S2)**. This difference suggests that large portion of p-lncRNAs could be post-transcriptionally regulated. Indeed, p-lncRNAs have been shown to be targeted by miRNAs in the cytoplasm as

they can act as competing endogenous RNA pools (Salmena *et al.*, 2011) and can also be affected by a plethora of degradation pathways in the cytoplasm (Houseley and Tollervey, 2009), while e-lncRNAs are less affected by these mechanism as they are rarely seen leaving the cell nucleus.

5.2.3 Regulation of super-enhancer activity upon hypoxia

Clusters of enhancers, also called super-enhancers (SE) have recently been recognized as some of the key players in gene regulation and cell identity maintenance and determination (Hnisz *et al.*, 2013; Lovén *et al.*, 2013; Whyte *et al.*, 2013; Mansour *et al.*, 2014) as well as cellular responses to stimuli (Brown *et al.*, 2014; Hah *et al.*, 2015; Michida *et al.*, 2020). SEs differ from typical enhancers by their association with multiple transcription factors, such as BRD4 and MED1, in the adjunct promoter regions that control phase separation which allow for a higher density of transcription factors locally (Sabari *et al.*, 2018; Nair *et al.*, 2019). Interestingly, SE target genes show higher fold change expression and absolute thresholds of expression than typical enhancers target genes that usually show lower fold change and graded responses (Spitz and Furlong, 2012; Lovén *et al.*, 2013; Whyte *et al.*, 2013). To study the changes in SEs profiles in HUVEC due to hypoxia, we analysed the normalized previously published H3K27ac signals of HUVEC under normoxic and hypoxic condition (Inoue *et al.*, 2014). By bioinformatically stitching regions within 12.5 kb of one another in the linear genome (Hnisz *et al.*, 2013; Lovén *et al.*, 2013; Whyte *et al.*, 2013), we identified SEs, which are defined as clusters of H3K27Ac signals. Next, their activity was assessed using GRO-seq as the signals detected correlate strongly with the enhancer's activity, as previously shown (Kim *et al.*, 2018; Mikhaylichenko *et al.*, 2018; Dong Wang *et al.*, 2011). Taken together, we identified 1058 and 799 SEs under normoxia and hypoxia conditions, respectively. Amongst those, 573 SEs were significantly regulated upon hypoxic conditions, with 385 SEs with induced activity and 215 repressed **(Manuscript II – Figure 3A and Supplementary Table S1)**.

It has been shown that SEs regulate proximal coding genes within a topologically associated domain (Dowen *et al.*, 2014). Therefore, we analysed

protein coding genes that were differentially expressed in hypoxia and located within the distance of 100 kb from induced and repressed super-enhancers in order to gain insight into their biological functionality. Interestingly, SE activity was induced in the vicinity of differentially expressed genes involved in angiogenesis such as *EGFL7* (Hong *et al.*, 2018), *DLL4* (Jin, Kaluza and Jakobsson, 2014), *MMP2* (Mahecha and Wang, 2017), and *TGFB1* (Sacilotto *et al.*, 2016). Additionally, SE activity induction was seen in the proximity of transcriptional activators such as *MEF2*, *SOX4* and *ETS1*. Differentially expressed genes found near SEs were also enriched for pathways such as regulation of apoptosis, regulation of NOTCH signalling, cell adhesion and, as expected, response to hypoxia. On the contrary, SE activity was decreased near genes encoding Rho GTPase-activating proteins such as angiogenesis inhibitors *ARHGAP7* (*DLC1*) (Shih *et al.*, 2010) and *ARHGAP18* (Chang *et al.*, 2014) as well as protein tyrosine phosphatase genes, for example, *PTPN14* and *PTPN1* (*PTP1B*), that have been shown to negatively regulate angiogenesis through the modulation of VEGF receptor signalling (Benzinou *et al.*, 2012; Lanahan *et al.*, 2014). SEs were found downregulated close to regulators of peptidyl-tyrosine de-phosphorylation, MAPK pathway activity as well as genes involved in signal transduction, endothelial cell migration and GTPase activity **(Manuscript II – Figure 3B, 3C and Supplementary Table S4)**. Strikingly, for 20% of the differentially expressed SEs, the closest transcript was a non-coding RNAs such as *MALAT1* that have been shown to regulate angiogenesis in earlier studies (Li *et al.*, 2016; Xuejing Zhang *et al.*, 2018) **(Manuscript II – Figure 3D)**.

Recent studies have demonstrated that SEs dynamic changes appear as a critical factor leading to the growth of blood vessels and more generally, the cellular and tissue adaptation to hypoxia (Kalna *et al.*, 2019; Mushimiyimana *et al.*, 2021; Sriram *et al.*, 2022). Further investigation of the modulation of cell response through the variation of SEs activity could offer new possibilities for therapeutic interventions. In this sense, studies have been obtaining promising results after targeting SEs, aimed to inhibit bromodomain and extra-terminal domain (BET) factors, which are a family of transcriptional co-activators and elongation factors, in order to modulate endothelial cell inflammatory responses (Brown *et al.*, 2014). Essentially, BET

modulation appears to be more efficient on SEs compared to that on typical enhancers, making it a good therapeutic approach directed towards modifying nearby gene expression (Lovén *et al.*, 2013).

5.2.4 Differentially expressed lncRNAs in human lesions

Multiple studies have stressed the importance of lncRNAs in cardiovascular development and pathophysiology (Schonrock, Harvey and Mattick, 2012; Grote *et al.*, 2013; Klattenhoff *et al.*, 2013) and as biomarkers associated with cardiovascular diseases (Kumarswamy *et al.*, 2014; Li *et al.*, 2016; Skroblin & Mayr, 2014; Vausort *et al.*, 2014). Therefore, to assess the expression of stable hypoxia-responsive lncRNAs *in vivo*, we turned our attention to their expression patterns in human atherosclerotic lesions. To this end, we used atherosclerotic segments of femoral arteries from 4 patients with primary atherosclerotic lesions, 5 with restenotic lesions and 4 control non-atherosclerotic arteries (Aavik *et al.*, 2015). Among the lncRNAs differentially expressed in hypoxia, 30 of them were also differentially expressed in the probe sets of the microarray of human lesions compared to the control tissue. By examining the expression signatures of these lncRNAs, we were able to distinguish between healthy and diseased vascular tissue highlighting their importance in cellular stress response **(Manuscript II – Figure 5)**. Among them, several lncRNAs, such as *HYMAI*, *KIAA1656*, *LOC339803* and *LOC730101* remain poorly studied and have not, so far, been associated with either atherosclerosis or hypoxia. Nonetheless, some interesting findings exist about these lncRNAs. *LOC730101* has been shown, in a previous study, to regulate the Wnt/β-catenin signalling pathway (Liu *et al.*, 2017) which is essential for both endothelial cell proliferation and migration (Reis and Liebner, 2013). Furthermore, *LOC730101* has been shown to promote cell survival through the AMP-activated protein kinase pathway during bioenergetic stress (Cheng *et al.*, 2018). *HYMAI* has previously been studied in relation to diabetes (Ma *et al.*, 2004; Mackay *et al.*, 2002) and has, more recently, been implicated in the dysregulation of imprinted genes which causes transient neonatal diabetes mellitus type 1 (Mackay & Temple, 2010). Furthermore, we identified *MALAT1* as one of the

most highly expressed and differentially expressed lncRNA. In our dataset, *MALAT1* is downregulated in restenotic and atherosclerotic lesions. *MALAT1* has been described to be involved in multiple cellular processes including alternative splicing (Tripathi *et al.*, 2010) and transcriptional regulation (Engreitz *et al.*, 2014). Moreover, *MALAT1* overexpression has previously been observed in cancer (Amodio *et al.*, 2018; Ji *et al.*, 2003), diabetes (Biswas *et al.*, 2018) and in response to a 24h exposure to hypoxia (Michalik *et al.*, 2014; Voellenkle *et al.*, 2016). Nevertheless, in line with our findings, other studies have described the downregulation of *MALAT1* in atherosclerotic plaques (Arslan *et al.*, 2017) demonstrating that the deregulation of *MALAT1* is dependent on the disease context (Li *et al.*, 2018). Besides, the mouse orthologous lncRNA *Malat1* was heterozygously ablated in that animal, it generated a massive immune system dysregulation and atherosclerosis due to the disruption of interactions between *Malat1*, *Neat1*, and key immune effector molecules (Gast *et al.*, 2019).

It is also noteworthy that two separate clusters appeared between restenotic samples following the intravascular surgery of the primary lesion and primary lesions indicating a further alteration of lncRNA profiles during intimal hyperplasia and arterial re-modelling in the treated arteries. In the clinic, this could provide improved insight into the disease progression and help pinpoint the activation of different types of cellular stress response.

In conclusion, this study provides a nascent transcriptome atlas of human hypoxia-sensitive lncRNAs in primary ECs that allowed the identification of lncRNAs which could be used as markers for endothelial hypoxia response in human atherosclerotic lesions or other vascular diseases. However, dissecting the functional roles of these lncRNAs warrants further studies.

5.3 PROFILING OF THE NUCLEAR AND CYTOPLASMIC NON-CODING TRANSCRIPTOMES IN ENDOTHELIAL CELLS UNDER HYPOXIA

5.3.1 Analysis of the coding transcriptome of fractionated cells compared to bulk RNA sequencing

The subcellular localization of lncRNAs is tightly regulated and necessary for their function (Chen, 2016). They can perform a wide variety of cellular functions, which are dependent on their cellular localization and the unique repertoire of molecules including proteins, RNA species, chromatin modifiers, with which they interact within each specific subcellular compartment. Depending on their site of action, lncRNAs participate in gene expression regulation near their transcription site (*cis*-regulation) or at a distance (*trans*-regulation) (Sun *et al.*, 2018; Yao *et al.*, 2019). In the nucleus, lncRNAs can control chromatin architecture, by recruiting chromatin modulating proteins as well as regulating inter-and intrachromosomal interactions. In manuscript III, we characterize the subcellular localization and regulation of lncRNAs in human primary ECs subjected to hypoxia.

The experiment began with a sub-cellular fractionation process, leading to the separation of cellular components into two distinct compartments: the nucleus and the cytoplasm. Following this, total RNA sequencing (RNA-seq) was performed on each compartment in order to analyze their RNA compositions. This method allowed for a detailed examination of both mRNAs and lncRNAs within these cellular environments, particularly focusing on the differences that arise under normoxic and hypoxic conditions.

Altogether, we identified 13892 and 17841 expressed transcripts, above the expression threshold of 0.5 cpm in at least a third of the samples, in the cytoplasm and in the nucleus, respectively. Amongst them, in the cytoplasm, 12684 were transcripts derived from protein-coding genes and 1208 were non-coding. In the nucleus, 14367 originated from protein-coding genes and 3474 were non-coding transcripts **(Manuscript III – Figure 1A)**. Overall, substantially more coding transcripts were identified in the different

subcellular compartments in comparison to non-coding transcripts. This discrepancy in abundance could be explained by a difference in RNA stability, as generally, lncRNAs and in particular nuclear non-coding RNAs are less stable than their coding counterparts. However, lncRNA stability is affected by a plethora of parameters such as their lengths, GC contents and genomic positions (Shi *et al.*, 2021). Moreover, the expression level of lncRNAs is usually lower than mRNAs and therefore detection could be affected by the expression threshold cut-off imposed (Mukherjee *et al.*, 2017).

However, looking at the difference in the lncRNAs expressed (count per millions above 0.5 in at least 3 samples) in each compartment separately, we identified a higher number of non-coding transcripts in the nuclear compared to the cytoplasmic compartment, which can be explained by a compartmental preference for the lncRNAs where they carry specific functions and/or regulatory roles. Alternatively, this could result from hypoxia-induced change in the rate of transcription (Tiana *et al.*, 2020). Supporting this, transcriptional activation has been suggested as the primary mechanism leading to induction of genes in response to hypoxia-mediated stabilization of HIF1α (Lum *et al.*, 2007; Kierans and Taylor, 2021) as exemplified by the activation of glycolytic genes (Lu *et al.*, 2002). Furthermore, HIF binding has been reported to increase transcription through the release of promoter-paused RNA pol II (Choudhry *et al.*, 2014). It is also worth noting that the number of differentially expressed lncRNAs, being similar between the two studied compartments, implies that the changes observed are most likely the results of the combination of both regulatory mechanisms.

We proceeded to examine the transcriptome changes upon hypoxic stimulation. Amongst the coding transcripts differentially expressed by at least a $\log_2$ fold change of above 1.5 or below -1.5, after a 24h exposure to hypoxia, we identified 318 and 238 coding transcripts in the cytoplasm and in the nucleus, respectively. Interestingly, gene ontology analysis revealed that the differentially expressed coding transcripts in the nucleus are associated with signalling pathways involved in hypoxia and tumour progression such as HIF1α and ID1 signalling pathways. Metabolic processes

such as glycolysis and neo-glucogenesis were also enriched, confirming the previous hypothesis regarding the increase in transcriptional rate through the action of HIF (Lum *et al.*, 2007; Tiana *et al.*, 2020; Kierans and Taylor, 2021). In addition, even though distinctive sets of genes are found differentially expressed in the cytoplasm, similar mechanisms are activated in the nuclear compartment, except for the inositol phosphate pathway that seems mostly cytoplasmic specific **(Manuscript III - Figure 1B)**.

A comparison between previously published bulk RNA-seq data and our subcellular RNA profiles in hypoxia-stimulated ECs shows drastically different genes **(Manuscript III - Figure 1C)**. This suggests that the compartment specific effects could be masked when RNAs from both compartments are analyzed as a pool. However, despite this variance, there is a noteworthy overlap in the enriched gene ontology pathways and biological processes **(Manuscript III - Figure 1D)** which suggests the involvement of similar regulatory mechanisms. In line with this, there is an enrichment of similar TFs between the different subcellular compartments and the bulk sequencing derived data, indicating their potential role in coordinating the expression of those DE genes within these pathways, and highlighting their significance in the observed pathway similarities **(Manuscript III – Supplementary Figure III)**.

Altogether, these results emphasize the implication of a core hypoxic response mechanism, alongside compartment-specific localization of lncRNAs, that might fine-tune the cellular response to hypoxia, highlighting the necessity of cellular fractionation in future studies. This is even more important in the case of lncRNAs that showed a consistent correlation between localization and function that is most likely due to changes in the composition of the subcellular microenvironment (Carlevaro-Fita & Johnson, 2019; Miao *et al.*, 2019; Yao *et al.*, 2019).

5.3.2 Compartmental profiling of non-coding RNAs

Long non-coding RNAs are expressed in a very cell type and stimulus specific manner and play important roles in a variety of physiological processes. Recently, experts specialized in the field of lncRNA research have

emphasized the importance of determining the lncRNA's subcellular localization as well as their dynamic stress condition responsiveness (Mattick *et al.*, 2023) as it is an essential step towards understanding their mechanisms of action in cells (Carlevaro-Fita and Johnson, 2019). In particular, the subcellular localization of these RNA molecules can determine their potential molecular interactions and, consequently, their biological functions. For example, a strictly nuclear specific lncRNA would most likely not be encoding for a short peptide since this phenomenon only occurs in the cytoplasm. Altogether, establishing the subcellular localization of lncRNAs plays a critical role in defining their functional relevance and their potential regulatory roles. By understanding how lncRNAs distribute within sub-cellular compartments and organelles, under specific stress conditions, we can gain valuable insights into their potential roles in cellular adaptation, response to stress, and overall gene regulatory networks (Djebali *et al.*, 2012; Bridges, Daulagala and Kourtidis, 2021).

Consequently, we focused on investigating the lncRNA enrichment in nuclear and cytoplasmic fractions by comparing the differential expression analysis of RNA-seq data between the two cellular compartments under identical conditions, namely normoxia and 24-hour of hypoxia. We categorized a lncRNA as belonging to a specific compartment if its expression exhibited a significant enrichment of at least 10-fold in one compartment over the other, and if the false discovery rate of the analysis was below 5% in similar conditions between the two compartments. In line with other studies, we identified an enrichment of lncRNAs in the nuclear compartment in comparison to the cytoplasmic compartment (Cabili *et al.*, 2015; Mas-Ponte *et al.*, 2017b) that could be explained by an increase in stability and function in the nucleus (Ransohoff, Wei and Khavari, 2018). Altogether we identified 1254 and 994 transcripts, respectively, in the nuclear compartment, under normoxia and hypoxia, of which 52% are shared between the conditions. At the same time, in the cytoplasmic compartment, we identified 47 and 53 transcripts under normoxic and hypoxic conditions, respectively. In this case, 66.7 % of these cytoplasmic lncRNAs are shared by both types of cellular exposure. In summary, we made first comprehensive mapping of lncRNAs in subcellular compartments

of ECs under normoxia and hypoxia **(Manuscript III - Figure 2A, 2B)**. Therefore, the high threshold selected enables the identification of highly enriched lncRNAs within the different subcellular compartments. Remarkably, less than 10% of these lncRNAs can be definitively assigned to a particular partition, challenging the prevailing understanding of their primarily nuclear-specific enrichment. Instead, these results imply a more diverse distribution across multiple compartments, which may be attributed to the dynamic interplay and translocation of these non-coding RNAs between different sub-cellular spaces.

The underlying mechanisms guiding the localization of lncRNAs are believed to reside in their sequence. The prevailing concept suggests that nuclear export is the default pathway and specific sequence elements are required for nuclear retention (Palazzo and Lee, 2018). Notably, certain nuclear lncRNAs harbour nuclear retention elements, and their deletion or mutation leads to cytoplasmic export (Miyagawa *et al.*, 2012; B. Zhang *et al.*, 2014; Lubelsky and Ulitsky, 2018). Additionally, those nuclear retention elements sequences alone do not solely determine whether a lncRNA will be localized to a distinct compartment, but it is further influenced by the availability and expression levels of other binding partners acting as trans-regulators (Schiene-Fischer, 2015). Ultimately, alternative splicing of lncRNAs adds another layer of complexity, as a single gene can give rise to multiple isoforms, each with its own unique subcellular localization patterns, influenced by specific retained or lost exonic regions (Fazal *et al.*, 2019; Zuckerman and Ulitsky, 2019; Guo, Xu and Chen, 2020). Localization of lncRNAs is also influenced by Pol II pausing and chromatin marks (Zuckerman and Ulitsky, 2019). Pol II promoter–proximal pausing is associated with increased nuclear export of lncRNA, and it is suggested that this pausing facilitates enhanced association with export factors (Zuckerman and Ulitsky, 2019). Additionally, epigenetic modifications, such as H3K27 acetylation and H3K4 di-/trimethylation, positively correlate with cytoplasmic enrichment, further emphasizing the roles of these modifications in lncRNA dynamics (Zuckerman and Ulitsky, 2019). Altogether, lncRNA localization is complex and directed by still-unknown factors. Indeed, analyzing these features as potential mechanisms

underlying subcellular localization of lncRNAs identified in this study, warrant further research.

5.3.3 Enrichment of disease-associated SNPs highlights the potential involvement of nuclear-enriched non-coding RNAs in cardiovascular diseases

Previously, genome-wide association studies (GWAS) have identified a large number of single-nucleotide polymorphisms (SNPs) associated with CAD (McPherson, 2014) that could eventually predict adverse cardiovascular events in diverse populations with and without pre-existing cardiovascular disease (Abraham *et al.*, 2016; Ganna *et al.*, 2013; Ripatti *et al.*, 2010; Vaara *et al.*, 2016). With this in mind, we interrogated the enrichment of SNPs in a ±50 kb window of compartment specific lncRNAs and hypoxia-deregulated lncRNAs and their association to CAD-risk factors.

Interestingly, the comparison between nuclear specific lncRNAs and endothelial specific lncRNAs showed that the lead SNPs in several CAD-associated categories, including “Coronary Artery”, “diastolic blood pressure”, “blood pressure,” and “Type 2 diabetes”, “Bone mineral density”, and ”waist to hip ratio”, are significantly located around or within detected lncRNAs that are nuclear-enriched in normoxia and hypoxia. In contrast, this enrichment was notably lower with regard to cytoplasmic-specific lncRNAs **(Manuscript III - Figure 3A and 3B)**. Altogether, these findings suggest that nuclear-specific lncRNAs may be hot spots of disease-associated regulatory variation and this could a major underlying cause of CVD within the human population.

5.3.4 nuclear-enriched lncRNAs regulate various cellular functions

Ultimately, we aimed to elucidate the functional role of candidate lncRNAs selected, based on their differential regulation by hypoxia, their enrichment in the nuclear compartment and the presence of SNPs within their vicinity.

In order to enhance the significance and originality of our findings, we designed our selection criteria based on several parameters. Firstly, we

focused on lncRNAs that demonstrated significant differential regulation in response to hypoxia, suggesting their potential involvement in hypoxia-responsive mechanisms. Secondly, we gave preference to lncRNAs primarily localized in the nucleus, aligning with our emphasis on regulatory processes centred around this cellular compartment. Thirdly, we prioritized lncRNAs that contained SNPs within their sequences, implying potential genetic influences on their functional roles. Lastly, to expand the extent of existing knowledge, we specifically searched for lncRNAs that had not been previously associated with either hypoxia or atherosclerosis. Employing these criteria, nuclear enriched *LUCAT1*, *SZT2-AS1* and *SLC2A1-AS1* that are upregulated and *A2M-AS1*, *SNHG15* and *SDCBP2-AS1* that are downregulated upon hypoxia, respectively, were selected for further study **(Manuscript III - Figure 4A)**.

Among the upregulated lncRNAs, *LUCAT1* has recently been linked to cardiovascular disease in cardiomyocytes. It has demonstrably protective effects on acute myocardial infarction (Xiao *et al.*, 2021), and in macrophages it has been shown to regulate the anti-inflammatory response programme by restraining the type I interferon (IFN) and the inflammatory response (Agarwal *et al.*, 2020). On the other hand, the function of *SZT2-AS1* is yet to be documented, although it has been observed to have increased expression in the subcutaneous tissue of metabolically unhealthy obese patients (Prashanth *et al.*, 2021). *SLC2A1-AS1* has been associated with cancer, specifically hepatocellular carcinoma, where it represses *GLUT1* expression through inhibition of the STAT3/FOXM1 pathway (Shang *et al.*, 2020). It has also been found to impact patient survival in lung adenocarcinoma (Qiu *et al.*, 2021).

Amongst the downregulated lncRNAs, *A2M-AS1* has been shown to attenuate hypoxic injury in cardiomyocytes (Song *et al.*, 2020; Yu *et al.*, 2022). *SNHG15*, which has previously and primarily studied in the context of cancer, holds promise as a prognostic and therapeutic target (Damaskos *et al.*, 2022). In addition, *SDCBP2-AS1* has been implicated in delaying ovarian cancer progression through miR-100-5p sponging (Liu *et al.*, 2021) and to reduce the proliferation of gastric cancer cells (Han *et al.*, 2022).

Additionally, the analysis of the genomic location of *SNHG15*, *SDCBP2-AS1*, *SZT2-AS1*, *SLC2A1-AS1* and *A2M-AS1* by the Cardiovascular Disease Knowledge Portal highlight the presence of multiple SNPs associated to cardiovascular diseases risks factors within their sequences implying that they might be implicated in CVD **(Manuscript III - Figure 4B)**. Indeed, how these SNPs could affect the function of these lncRNAs is still unknown however, but it is reasonable to suggest that single base substitutions could induce structural changes in the lncRNA, which in turn could affect its physical stability and/or their interaction of with other RNA or macromolecules.

To interrogate the role of these non-coding RNAs in hypoxic and normoxic conditions, HUVEC cell cultures were transfected with antisense oligonucleotides (ASOs) knocking down *LUCAT1*, *SZT2-AS1*, *SLC2A1-AS1*, *A2M-AS1*, *SNHG15* and *SDCBP2-AS1* and then exposed for 24h to hypoxia or kept in normoxic condition **(Manuscript III - Figure 4C and Supplementary Figure VI)**. ASOs were selected for their superior efficacy over synthetic siRNAs in targeting nuclear-localized lncRNAs (Lennox and Behlke, 2016). Subsequently, the bulk RNA transcriptome was sequenced to determine the potential targets genes of those non-coding RNAs, alongside the cellular functions they could be involved in, by investigating the gene sets against pathway ontology databases.

Our analysis of the controls in hypoxic and normoxic conditions confirmed the enrichment of "HALLMARK_HYPOXIA" in hypoxic samples. Interestingly, following singular knock-downs of *SDCBP2-AS1*, *A2M-AS1* and *SZT2-AS1*, we noticed a decrease in genes involved in "HALLMARK_HYPOXIA", indicating a role for these non-coding RNAs in enhancing the hypoxic response **(Manuscript III - Figure 4C)**.

We also examined gene sets associated with cell proliferation, such as "HALLMARK_E2F_TARGETS", "HALLMARK_G2M_CHECKPOINT", "HALLMARK_MYC_TARGETS_V1", and "HALLMARK_MYC_TARGETS_V2". These pathways were underrepresented in controls exposed to hypoxia, compared to normoxia. Yet, after suppressing certain non-coding RNAs (*SDCBP2-AS1*, *A2M-AS1, SLC2A1-AS1, SZT2-AS1* and *LUCAT1*), we observed an enrichment of these pathways, suggesting these RNAs contribute towards the regulation of cell proliferation in HUVECs **(Manuscript III - Figure 4C)**.

Moreover, our findings suggest that *A2M-AS1*, *SNHG15*, and *SDCBP2-AS1* might play a key role in the regulation of inflammation. This was suggested by their involvement in the "HALLMARK_INTERFERON_GAMMA_RESPONSE" in normoxia for *SNHG15* as well as *A2M-AS1*, and in "HALLMARK_TNFA_SIGNALING_VIA_NFKB" in hypoxia for *SDCBP2-AS1*. *SZT2-AS1*'s role in controlling inflammatory response in HUVEC is highlighted by the under representation of genes in the "HALLMARK_TGF_BETA_SIGNALING" upon its knock-down in hypoxic conditions **(Manuscript III - Figure 4C)**.

In addition, we looked into metabolic reprogramming, a common adaptation to hypoxic conditions in most eukaryotic cells, involving an increase in the rate of glycolysis (Kierans and Taylor, 2021). In our hypoxic controls, compared to normoxic ones, the "HALLMARK_GLYCOLYSIS" gene set was positively enriched. However, this set was negatively enriched when *SDCBP2-AS1*, *SLC2A1-AS1* and *SZT2-AS1* were knocked down specifically under hypoxic conditions, signifying their role in the metabolic adjustment of cells to hypoxia **(Manuscript III - Figure 4C)**.

In conclusion, our results suggest that these non-coding RNAs (*SDCBP2-AS1, A2M-AS1, SLC2A1-AS1, LUCAT1,* and *SZT2-AS1*) have significant roles in HUVEC proliferation under hypoxia, in addition to their role in regulating inflammatory gene responses and glycolysis. Nonetheless, the different hallmarks affected point to varied regulatory mechanisms for these functions.

5.3.5 Nuclear-enriched lncRNAs coordinate endothelial cell proliferation

To validate the implication of the selected lncRNAs in proliferation, we assessed the proliferative capabilities of HUVEC cultures following transfection with ASOs to knock-down *SDCBP2-AS1, A2M-AS1, SLC2A1-AS1, LUCAT1, SNHG15* and *SZT2-AS1*. Proliferation was evaluated through fluorescence quantification, following 24 hours of hypoxia, and comparisons were made with ASO control transfection.

According to our RNA-seq results, *SNHG15*, *SDCBP2-AS1* and *A2M-AS1* are naturally downregulated by hypoxia in ECs suggesting their involvement in proliferation under normal conditions. Surprisingly, the knock-down of *A2M-AS1* and *SNHG15* in normoxia and hypoxia showed an increasing rate of proliferation in treated HUVEC cells. One important consideration is that the use of ASOs might have further decreased the expression levels of *SNHG15* and *A2M-AS1*, contributing to the observed effects.

Conversely, *SLC2A1-AS1* was upregulated upon hypoxia stimulus. Intriguingly, the knockdown of *SLC2A1-AS1* under hypoxic conditions resulted in increased proliferation, emphasizing its potential as a proliferation inhibitor in HUVECs. In contrast, *LUCAT1*, which is naturally expressed at high levels and further enhanced under hypoxic conditions, exhibited a marked reduction in proliferation when knocked down, both in normoxic and hypoxic conditions. These findings underscore the significant role of *LUCAT1* as a major driver of endothelial cell proliferation under hypoxic conditions.

Overall, this experiment highlights the intricate interplay between lncRNAs and their targets, as the knock-down of naturally downregulated lncRNAs in hypoxia can elicit opposite effects depending upon the stimulus. This disparity may arise from alterations in the cellular microenvironment triggered by hypoxia, which in turn affect the availability of lncRNA targets. Moreover, it is important to consider that the observed differences are a result of both natural downregulation and ASO-mediated repression. Thus, careful interpretation of the findings is warranted, and additional experiments, such as induction studies, may be required to validate the observed effects.

In conclusion, the subcellular localization of lncRNAs emerges as an additional essential layer of gene regulation affecting cellular function. In addition, determining the subcellular localization of lncRNAs plays a crucial role in deciding the appropriate targeting strategy, whether it's through RNA interference (RNAi) or antisense oligonucleotides to target cytoplasmic or nuclear lncRNAs, respectively (Lennox and Behlke, 2016). Here, we unveil the complex expression patterns of lncRNAs within the nuclear and cytoplasmic compartments of HUVECs in culture, shedding light on their

dysregulation under hypoxic stress. Our study has some limitations. We acknowledge that biochemical fractionation may be biased in its representation of subcellular compartment as our experiment was performed on unsynchronized cell cultures which will contain a subpopulation that is undergoing mitosis which release their nuclear RNAs as the nuclear membrane breaks down (Carlevaro-Fita and Johnson, 2019). Our findings still provide a valuable sequencing resource for researchers exploring compartment-specific expression in ECs, but also offer crucial insights into the molecular response to hypoxia and the role and localization of lncRNAs in human atherosclerotic lesions and other cardiovascular diseases. These findings position lncRNAs as promising indicators of endothelial hypoxia and as potential therapeutic or prognostic targets. Future investigations delving into the precise mechanisms underlying the regulation and action of these lncRNAs are eagerly anticipated, paving the way for a deeper understanding of their functional roles and transformative applications in the field of cardiovascular research. Future studies should consider employing long-read sequencing to identify specific sequence elements, and to explore the impact of alternative splicing on the diversity of lncRNA isoforms, both of which governing subcellular localization.

6 CONCLUSIONS

I. Despite the cell-type-specific expression of multi-variant primary miRNAs, most mature miRNAs are common across different cell types, and they predominantly consist of a few abundant species.

II. The microRNA miR-100-5p participates in the inhibition of the HIPPO pathway in endothelial cells, leading to the activation of the YAP/TAZ cascade. Additionally, miR-100-5p plays cell type specific regulatory roles by enhancing genes involved in TNFα, IFNβ, and hypoxia-induced pathways in smooth muscle cells and suppressing these genes in endothelial cells.

III. Hypoxia regulates the nascent transcription of ~1800 lncRNAs. Amongst them, promoter-associated lncRNAs are more likely to be induced by hypoxia, compared to enhancer associated lncRNAs, which could be attributed to differential activities of HIF1α and HIF2α and the binding of distinct transcription factors upon hypoxia stimulus. Furthermore, hypoxia leads to a significant induction in the activity of super-enhancers next to genes and transcription factors implicated in angiogenesis, cell survival and adhesion.

IV. Many of the lncRNAs identified *in vitro* were also differentially regulated *in vivo* in primary and restenotic human atherosclerotic lesions making them potential biomarkers for lesion hypoxia.

V. Exposure to hypoxia induces compartment-specific deregulation of gene expression, implicating distinct molecular pathways. In particular, lncRNAs, predominantly localized in the nucleus,

exhibit differential expression alterations due to hypoxia, contingent upon the subcellular compartment. Notably, only 23% of lncRNAs demonstrate deregulation in both the nucleus and cytoplasm, underscoring the impact of hypoxia on subcellular RNA dynamics.

VI. The nuclear-enriched lncRNAs *SDCBP2-AS1*, *A2M-AS1*, *SLC2A1-AS1*, *LUCAT1*, *SNHG15* and *SZT2-AS1* are deregulated in hypoxia and their knock-down highlights their importance in the regulation of immune response and endothelial cell proliferation.

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ORIGINAL PUBLICATIONS (I – III)

I

Profiling of Primary and Mature miRNA Expression in Atherosclerosis-Associated Cell Types

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Arteriosclerosis, Thrombosis, and Vascular Biology 41:2149–2167, 2021

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Arteriosclerosis, Thrombosis, and Vascular Biology

BASIC SCIENCES

Profiling of Primary and Mature miRNA Expression in Atherosclerosis-Associated Cell Types

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OBJECTIVE: Atherosclerosis is the underlying cause of most cardiovascular diseases. The main cell types associated with disease progression in the vascular wall are endothelial cells, smooth muscle cells, and macrophages. Although their role in atherogenesis has been extensively described, molecular mechanisms underlying gene expression changes remain unknown. The objective of this study was to characterize microRNA (miRNA)-related regulatory mechanisms taking place in the aorta during atherosclerosis.

APPROACH AND RESULTS: We analyzed the miRNA expression changes in primary human aortic endothelial cells and human umbilical vein endothelial cells, human aortic smooth muscle cells, and macrophages (CD14+) under various proatherogenic stimuli by integrating GRO-seq, miRNA-seq, and RNA-seq data. Despite the highly cell-type-specific expression of multivariant primary miRNAs, the majority of mature miRNAs were found to be common to all cell types and dominated by 2 to 5 abundant miRNA species. We demonstrate that transcription contributes significantly to the mature miRNA levels although this is dependent on miRNA stability. An analysis of miRNA effects in relation to target mRNA pools highlighted pathways and targets through which miRNAs could affect atherogenesis in a cell-type-dependent manner. Finally, we validate miR-100-5p as a cell-type specific regulator of inflammatory and HIPPO-YAP/TAZ-pathways.

CONCLUSIONS: This integrative approach allowed us to characterize miRNA dynamics in response to a proatherogenic stimulus and identify potential mechanisms by which miRNAs affect atherogenesis in a cell-type-specific manner.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: atherosclerosis ■ cardiovascular diseases ■ endothelial cells ■ gene expression ■ macrophages ■ microRNA

Early events in atherogenesis take place in the endothelium, which forms the inner surface of the vascular wall (Figure IA in the Data Supplement). Endothelial dysfunction increases the vascular permeability promoting lipid accumulation into the vessel wall, and increases local oxidative stress leading to the formation of oxidized phospholipids, which have been shown to accumulate in the atherosclerotic plaques.[1] The formation of oxidized lipids stimulates chemokine production, which attracts monocytes to the site, and induces their differentiation into macrophages. After the ingestion of oxidized lipids, macrophages turn into foam cells that perpetuate vascular remodeling. Eventually, the local release of inflammatory mediators induces proliferation and migration of vascular smooth muscle cells, resulting in vessel wall thickening and hypoxia, which further stimulates intraplaque angiogenesis and facilitates the progression of the disease.[1,2] Although the general proatherogenic

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The Data Supplement is available with this article at https://www.ahajournals.org/doi/suppl/10.1161/ATVBAHA.121.315579.

For Sources of Funding and Disclosures, see page 2164.

Nonstandard Abbreviations and Acronyms

CD14+	macrophages
DGCR8	DiGeorge syndrome critical region 8
GRO-seq	global run-on sequencing
HAECs	human aortic endothelial cell
HASMCs	human aortic smooth muscle cell
HRP	horseradish peroxidase
HUVECs	human umbilical vein endothelial cell
miRNA	microRNA
oxPAPC	oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine
PCR	polymerase chain reaction
pre-miRNA	precursor miRNA
pri-miRNA	primary microRNA
TF	transcription factor
TSS	transcriptional start site
TV	transcript variant

Highlights

- Pri-miRNAs (primary-microRNAs) transcription and transcriptional start site usage is highly cell-type specific and minimally affected by the proatherogenic stimulus.
- Mature miRNA expression patterns are similar among cells types and poorly correlated to primary miRNA expression. Furthermore, 2 to 5 miRNAs are shown to account for over 50% of the total miRNA expression in the cells.
- Pri-miRNA expression under single and combined proatherogenic stimulus provide evidence of similar transcription factor enrichment at regulated transcriptional start sites.
- miR-100-5p silences the HIPPO pathway, leading to the activation of the YAP/TAZ cascade in endothelial cells.
- TNFα-, IFNβ-, and hypoxia-pathway mediators are positively enriched among the miR-100-5p regulated genes in human aortic smooth muscle cells but negatively enriched in human umbilical vein endothelial cells. Similarly, miR-100-5p regulated genes in human umbilical vein endothelial cells were enriched for genes involved in proliferation whereas in human aortic smooth muscle cells the genes were enriched for apoptosis.

changes in endothelial cell, macrophage, and smooth muscle cell function have been described, the mechanisms leading to these changes have not been characterized. This is mainly due to a lack of knowledge on how transcriptional programs differ between cell types and a lack of comprehensive gene expression data which integrates different levels of gene regulation.

MicroRNAs (miRNAs) are small noncoding RNAs that repress gene expression post-transcriptionally in the cytoplasm[3] but may also mediate noncanonical roles to regulate gene expression in the nucleus,[4] as well as protein function.[5] miRNA biogenesis begins with primary miRNA (pri-miRNA) transcription from the genome, followed by cleavage with the DGCR8 (DiGeorge syndrome critical region 8)/Drosha complex into a hairpin-shaped loop structure called precursor-miRNA (pre-miRNA; Figure IC in the Data Supplement). Pre-miRNA is exported from the nucleus to the cytoplasm and further processed by Dicer into 2 single-stranded mature miRNAs.[6] The primary role of miRNAs is to fine-tune cellular functions and maintain tissue homeostasis, but in pathological states their effects become more pronounced and play more decisive roles.[6] miRNA therapeutics that manipulate cellular miRNA levels have already entered clinical trials,[6] although many aspects of miRNA function remain elusive.

Knowledge on the roles that miRNAs play in gene regulatory networks has increased significantly in the past 2 decades following the initial miRNA discovery.[7,8] However, most profiling studies have been performed at the tissue/organ level, which makes it difficult to discern the actual causes of the altered signals between different tissue types and patient and control samples. Tissues are heterogeneous collections of cell types and the observed changes in the miRNA profiles can, for instance, arise from the differences in the cell composition of the samples rather than from changes in miRNA expression in specific cell types.[9] Similarly, the miRNA expression arising from cell types that are found essentially in all organs, such as endothelial cells, red blood cells, and fibroblasts, can be misinterpreted as ubiquitous without knowledge on cellular miRNA expression profiles.[10] Having cell-type-specific knowledge on miRNA expression patterns assures that functional miRNA studies are performed in appropriate cell types and will thus be of biological relevance.

Recent efforts[11–13] to clarify the global miRNA expression patterns in human primary cells are filling the gaps in present knowledge, but the cellular miRNA expression patterns under disease-causing stimuli remain unknown. Although both miRNA and mRNA profiling studies have actively been conducted in various disease-relevant contexts, integration of different levels of genomics data is rarely done to elucidate miRNA expression and function. Importantly, mature miRNAs are processed from primary transcripts (pri-miRNAs), and their expression is controlled at the transcriptional and posttranscriptional levels. However, how regulation at multiple levels achieves precise control remains elusive.

In this study, we aimed to address these questions by investigating the miRNA expression profiles in atherosclerosis-modeling primary cell types, namely human

aortic endothelial cell (HAEC) and human umbilical vein endothelial cell (HUVECs), aortic smooth muscle cells (HASMCs), and macrophages (CD14+), subjected to proatherogenic stimuli (Figure IB in the Data Supplement) to shed light on the miRNA-related regulatory mechanisms that could contribute to atherosclerosis. To achieve this, we generated transcriptomics data sets measuring nascent primary miRNA transcription (global run-on sequencing [GRO-seq]), mature microRNAs (miRNA-seq), and target mRNAs (ribosome depleted RNA-seq; Figure IC in the Data Supplement). Using this integrative approach, we were able to establish regulatory networks among the proatherogenic-stimuli-responsive miRNAs and their target genes and to identify potential players driving proatherogenic changes in a cell-type-specific manner.

MATERIALS AND METHODS

Cell Cultures and Treatments

HUVECs were isolated from 6 different donors. HUVECs were extracted from umbilical cords obtained from the maternity ward of Kuopio University Hospital (used at passage 4–7) or purchased from Lonza (passage 9). This study has been performed according to the recommendations of the Research Ethics Committee of the Hospital District of Northern Savo, Kuopio, Finland. Informed written consent was received from all the participants, and the experiments were performed according to the relevant guidelines and regulations. HUVECs were cultivated in Endothelial Cell Basal Medium (EBM; Lonza) with recommended supplements (EGM SingleQuot Kit Supplements and Growth Factors, Lonza) after a fibronectin-gelatin coating (10 μg/mL fibronectin [Sigma, St Louis, MO] and 0.05% gelatin). Each HUVEC donor was analyzed as a separate replicate.

HAECs from different donors were obtained from Lonza and cultivated in Endothelial Cell Basal Medium (Lonza) with recommended supplements (EGM SingleQuot Kit Supplements & Growth Factors, Lonza) after fibronectin-gelatin coating (10 μg/mL fibronectin [Sigma, St Louis, MO] and 0.05% gelatin). HASMCs from different donors were obtained from Lonza and cultivated in medium 231 (Thermofisher, Carlsbad, CA) supplemented with smooth muscle growth supplement. Human monocytes from different donors were obtained from Lonza. The monocytes were cultivated in Roswell Park Memorial Institute (RPMI) 1640 Medium, supplemented with 10% FBS, 100 μg/mL streptomycin, 100 U/mL penicillin, 2 mmol/L glutamine, 1% Na-pyruvate, 1% NEAA (Non-Essential Amino Acid cell culture supplement), and supplemented with rHu M-CSF (50 ng/mL; ThermoFisher Scientific) to differentiate them into macrophages. The monocyte-derived macrophages will be referred to as CD14+ macrophages throughout the study.

To mimic an intermediate atherosclerotic state, we treated cells with oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (oxPAPC) and hypoxia for 7 hours. Oxidized phospholipids have been reported to accumulate after disruption of the endothelial barrier, contributing to vascular inflammation.[14–17] In addition, hypoxia is present in atherosclerotic lesions stimulating proatherosclerotic processes.[18] A 7-hour timepoint was chosen to capture both early and late transcriptional responses to stimulus.[19] Moreover, short exposure to hypoxia tends to promote cell survival and growth, while prolonged exposure to hypoxia leads to cell death.[20] Hypoxia was achieved using a Ruskinn InvivO2 400 hypoxia workstation (Baker Ruskinn, Bridgend, Wales) in the presence of 1% O_2 and 5% CO_2. OxPAPC was generated from 1-palmitoyl-2-archidonoyl-sn-glycero-3-phophocholine (PAPC, 10 mg/mL [Avanti Polar Lipids Inc, Alabaster, AL]). PAPC was exposed to air for 40 hours, then dissolved in chloroform and stored at −70 °C. At the start of the oxPAPC stimulation, chloroform was evaporated with nitrogen gas, and lipids were resuspended in growth medium to achieve a concentration of 30 μg/mL.

GRO-Seq, RNA-Seq, and miRNA-Seq

For the GRO-seq, HUVECs, HAECs, HASMCs, and CD14+ macrophages were treated with a medium containing 1% FBS with or without oxPAPC (30 μg/mL) for 6 hours under normoxia or hypoxia. Cells were washed twice with cold PBS and incubated on ice with 10 mL of swelling buffer (10 mmol/L Tris-HCl, 2 mmol/L MgCl2, 3 mmol/L CaCl2, and 2 U/mL SUPERase inhibitor [Thermofisher, Carlsbad, CA]) for 5 minutes. The cells were scraped and centrifuged at 400*g* for 10 minutes and resuspended in 50 μL of swelling buffer supplemented with 10% glycerol. Next, 500 μL of swelling buffer containing 10% glycerol and 1% Igepal was added drop by drop while gently vortexing. The nuclei were washed twice with a lysis buffer (10 mL of swelling buffer containing 0.5% igepal and 10% glycerol), and once with 1 mL of a freezing buffer (50 mmol/L Tris-HCl pH 8.3, 40% glycerol, 5 mmol/L MgCl2, and 0.1 mmol/L EDTA). Finally, the nuclei were counted, centrifuged at 900*g* for 6 minutes to be further resuspended into a concentration of 5 million nuclei per 100 μL of freezing buffer, snap-frozen in liquid nitrogen, and stored at −80 °C until run-on reaction. The run-on reaction and library preparation were performed as described in Bouvy-Liivrand et al.[21] Libraries were amplified using 13 to 15 cycles, size-selected (180–350 bp) from 10% TBE gels (Life Technologies) and sequenced (single end 50 bases) using an Illumina HiSeq 2000 at EMBL GeneCore (Heidelberg, Germany).

RNA-seq samples were obtained 1 hour after the collection of the GRO-seq samples from the matching samples (reflecting the lag in nascent transcription and mature RNAs). The cells were treated for 10 minutes with cycloheximide (0.1 mg/mL) to stop mRNA translation,[22–24] subsequently washed with PBS and scraped into a lysis buffer (1x Mammalian Polysome Buffer [Epicentre, Madison, Wisconsin], 1% Triton X-100, 1 mmol/L DTT, 250 U/mL SUPERase Inhibitor, 7.1 U/ml Turbo DNase [ThermoFisher Scientific, Waltham, MA] and 0.1 mg/mL Cycloheximide) on ice. To confirm the complete lysis of the cells, they were drawn up and expelled 4× through a sterile 22 to 25 gauge needle. The cleared whole cell lysate was further treated with 10% SDS, snap-frozen in liquid nitrogen, and stored at −80 °C. Larger mRNAs (>200 nt), from the total RNA, were purified with a Zymo RNA Clean and Conc kit (Zymo Research, Irvine, CA), and rRNAs were eliminated using the Ribo-Zero Gold rRNA Removal Kit (Illumina, San Diego, CA). The RNA was then fragmentated (RNA fragmentation reagent, Thermofisher) and dephosphorylated. The libraries were prepared as described in Bouvy-Liivrand et al[21] for

GRO-seq but omitting the anti-BrUTP pulldown. The libraries were amplified by 11 to 16 cycles, size selected (190–350 bp), and quantified (Qubit dsDNA HS Assay Kit on a Qubit fluorometer, Thermofisher, Carlsbad, CA). Sequencing (single-end 50 bases) was performed with an Illumina Hi-Seq2000 at EMBL GeneCore (Heidelberg, Germany).

For the RNA-seq gene expression quantification of the HASMC and HUVECs samples transfected with miRNA mimic control and miR-100-5p treated with hypoxia+oxPAPC, the total RNA was isolated using an RNeasy Mini Kit (QIAGEN). Libraries were prepared using the QuantSeq 3′ mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen) according to the manufacturer's instructions. For each sample, 250 ng of total RNA was used for the library preparation, and the libraries were sequenced using a read length of 78 bases (single-end) on an Illumina NextSeq 500 sequencer. The average sequencing depth was 6.3×10^6 reads per library (range, $5.5–7.3\times10^6$).

A small RNA-fraction (17–200 bases) was obtained by adding ethanol to the flow through from the total RNA extracted from the RNA Clean and Concentrator (Zymo Research, Irvin, CA) kit and passing the RNA through a new column. The libraries were prepared at the Finnish Microarray and Sequencing Centre Turku Centre for Biotechnology (Turku, Finland) using the TruSeq small RNA-seq protocol or at Exiqon (Vedbæk, Denmark) with the small RNA-Seq protocol.

miRNA Overexpression and Silencing (Transfection)

HUVECs were seeded on 6-well plates and transfected at 70% confluence using Oligofectamine (Invitrogen, Carlsbad, CA). The following oligonucleotides were employed: MISSION miRNA Mimics Negative Control No. 1 (HMC0002, Sigma-Aldrich, Saint-Louis, MO), hsa-miR-100-5p mimic (HMI0023, Sigma-Aldrich, Saint-Louis, MO), MISSION Synthetic microRNA (miRNA) Inhibitors Negative control I (NCSTUD001, Sigma-Aldrich, Saint-Louis, MO), and hsa-miR-100-5p inhibitor (HSTUD0023, Sigma-Aldrich, Saint-Louis, MO). Mimic miRNAs were used at a final concentration of 25 nmol/L, while inhibitor miRNAs were used at 1 nmol/L. Medium supplements were added 4 hours posttransfection, and on the next day the cells were washed with PBS and a fresh EBM medium with full supplements was added. RNA isolation, Western blot, and miRNA expression analyses were performed 48 hours after transfections.

miRNA Purification

HUVEC miRNAs and mRNAs were separated using an RNeasy Mini Kit (QIAGEN, Hilden, Germany). The cells were disrupted using 350 µL of buffer RLT, followed by 350 µL of 70% ethanol. The samples were then transferred to an RNeasy Mini spin column and centrifuged for 15 s at 8000*g*. The flow through was kept to extract the miRNAs while the column was loaded with the larger RNAs (>200 nt). The miRNA fraction was diluted using a 0.65 volume of 100% ethanol and loaded into a new RNEasy Mini Kit (QIAGEN, Hilden, Germany) column. The column was then washed using 2 steps of RPE buffer (RNEasy Mini Kit [QIAGEN, Hilden, Germany]) of 700 µL and 500 µL, respectively, followed by 500 µL of 80% ethanol. Each washing step was separated by a centrifugation step at 8000*g*, and the flow through was discarded. The miRNAs were then eluted in RNAse-free water.

miRNA cDNA Synthesis

The miRNA fraction was reverse transcribed using a miRCURY LNA Universal microRNA polymerase chain reaction (PCR; Exiqon, Vedbaek, Denmark) kit according to manufacturer's protocol for individual assays.

Quantitative PCR Analysis

A reverse-transcribed miRNA fraction was diluted 80-fold in RNAse-free water and measured in 10 µL PCR reactions according to the protocol for the miRCURY LNA Universal microRNA PCR (Exiqon, Vedbaek, Denmark) and the ExiLENT SYBR Green master mix (Exiqon, Vedbaek, Denmark) using a LightCycler 480 Real-Time PCR System (Roche, Basel, Switzerland). Levels of the miRNAs were determined using a miRCURY LNA miRNA PCR assay primer mix (Exiqon, Vedbaek, Denmark) of miR-100-5p and *SNORD48*. Data analysis was done using the Roche LC software for Cp determination (using the second derivative method) and for a melting curve analysis. *SNORD48* was used for the normalization of the miRNA levels.

For mRNA detection upon miR-100-5p silencing and overexpression, quantitative PCR was performed on the Applied Biosystems StepOne Plus TM system using SYBR Green ER master mix (Invitrogen) and the following conditions: 10 minutes at 95 °C, then 40 cycles of 15 seconds at 95 °C, 15 seconds at 60 °C, and 30 seconds at 72 °C. The *RPLP0* and *ATP5F1* housekeeping genes were used for the normalization of the mRNA levels. Primer sequences used are listed in Table I in the Data Supplement. Data was checked for normal distribution before performing statistical tests. Paired Student *t* test (2-tailed) was used for data that followed normal distribution and equal variance. Otherwise, nonparametric Mann-Whitney test was used. $P<0.05$ was used to define a significant difference between the groups.

Western Blot Sample Preparation

HUVEC samples were collected using an NE-PER Nuclear and Cytoplasmic Extraction kit (ThermoFisher Scientific, Waltham, MA) following the manufacturer's protocol for an adherent cell culture. A PhosSTOP Phosphatase Inhibitor Cocktail (Roche, Basel, Switzerland) and cOmplete, EDTA-free Protease Inhibitor Cocktail (Roche, Basel, Switzerland) were used to replace the BupH Phosphate (ThermoFisher Scientific, Waltham, MA), and the Thermo Scientific Halt Protease Inhibitor Cocktail (ThermoFisher Scientific, Waltham, MA), respectively. Protein concentrations were assessed using a Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA) according to manufacturer's protocol. Equal amounts of proteins were loaded into stain-free gels (Bio-Rad, Hercules, CA) from each sample (10 µg of nuclear and 15 µg of cytoplasmic proteins in HUVEC and 5 µg of proteins in all HASMC samples). For total protein measurement, the stain-free gel was activated with 2.5 minutes exposure to UV-light before protein transfer to membrane. For detecting and normalizing the amount of nuclear TAZ (transcriptional co-activator with PDZ-binding motif) protein, primary antibodies YAP (Yes-associated protein)/TAZ and

histone H3 (Cell Signaling Technology, Danvers, MA) were used. Similarly, antibodies for phospho-TAZ and β-actin (Cell Signaling Technology, Danvers, MA) were used to determine the cytoplasmic retention of TAZ. HRP (Horseradish peroxidase) conjugates were used as secondary antibodies. Detection of antigen-antibody complexes was performed with a PIERCE ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA) and ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA). A quantitative analysis of the immunoblot target protein bands were performed with ImageLab 6.0 software (Bio-Rad, Hercules, CA). For HUVEC samples, the adjusted volume intensity values of target proteins were normalized with the same values of the loading controls (Histone H3, β-actin). For HASMC samples, the target protein intensity values were obtained with an automatic total protein normalization tool. The loading control balanced values were normalized to the respective treatment control.

Data Analysis

Reads obtained after library preparation were poly(A) trimmed and low-quality reads were discarded. After the quality control, the miRNA reads were trimmed to a final size of 21 bp. For GRO-seq, reads passing quality control were mapped to the hg19 genome using Bowtie.[25] The Bowtie parameters allowed up to 2 mismatches and reporting only one alignment for each read. RNA-seq and miRNA-seq reads were aligned to the GRCh37/hg19 reference genome using STAR v2.5.4b.[26] STAR parameters followed ENCODE standard options for a long RNA-Seq pipeline and small RNA-Seq pipeline.[27] Tag directories were generated with the fragment length set to 75 and for the RNA-seq, the maximum number of tags per base pair was set to 3. Raw counts were quantified using Homer V4.9[28] and the analyze Repeats routine using the standard option, or a customized bed file containing the annotations from mirbase v22[29–31] and the custom pri-miRNA coordinates (Table III in the Data Supplement).[21] The fold change was calculated after filtering low expressed transcripts (RPKM >0.5 in at least 3 samples) for each cell type to improve the sensitivity and the precision of the differential expression analysis.[32] Differential expression was calculated using EdgeR[33,34] or DESeq2[35] for each cell type individually.

For RNA-seq gene expression quantification of HASMC and HUVEC samples transfected with miRNA mimic control and miR-100-5p, reads were first trimmed for poly(A), Illumina adapter, and low-quality bases using cutadapt (version 2.8[36]). Subsequently, the nf-core RNA-Seq pipeline (version 1.4.2[37]) was used to align the reads to the GRCh37/hg19 human genome with the STAR aligner and count the reads in transcripts according to the Ensembl GRCh37 release 87 gene annotations. The following gene biotypes were retained in the gene expression matrix: protein coding, lincRNA, and antisense. To filter out lowly expressed genes, the filterByExpr function of the EdgeR package (version 3.24.3[33,34]) was used (minimum count: 5; minimum total count: 15). For each cell type, all genes were ranked by the response to miR-100-5p (relative to mimic control) based on the Wald statistic calculated by the DESeq2 package (version 1.22.2[35]) using the default parameters. Gene set enrichment analysis of the ranked lists was performed using the fgsea package (version 1.8.0[38]) with the Hallmark gene sets obtained from the Molecular Signatures Database (release 7.1[39]).

Pri-miRNA Transcript Variant Annotation and Quantification

Primary miRNA transcript coordinates were identified from GRO-seq data using a custom de novo detection pipeline.[21] To achieve this, GRO-seq, and matching ChIP-seq and CAGE-seq data were assembled for 27 human cell types. Briefly, nonmappable coordinates, exons of coding genes, and ribosomal RNA regions were removed from pri-miRNA transcripts before quantification using BEDTools[40] (subtractBed) to exclude regions known to cause problems in quantification of GRO-seq data.

Next, primary transcripts were identified de novo from GRO-seq nascent transcripts using the HOMER software suite program findPeaks.pl, with the -groseq -uniqmap options, as well as 3 parameter settings for varying detection sensitivity and specificity. Transcription start site (TSS) coordinates were then assigned based on CAGE-seq peaks within ±500 bp of the detected transcripts and collapsed for unique across cell types. The TSS status was further supported by promoter histone methylation levels: all putative TSSs had to exhibit >10 CPM H3K4me3 and 5-fold H3K4me3> H3K4me1. Transcript end coordinates were assigned based on de novo transcripts clustered by adjacency and a change point analysis. The final de novo coordinates represent nonoverlapping regions between TSSs across individual loci thus allowing the investigation of transcription originating from different TSSs per locus. Pre-miRNA locations were assigned based on GENCODE (v19) and miRBase (v20) existing annotations and subsequently the overlap of de novo coordinates was used to define candidate pri-miRNA transcripts. To obtain transcript variant (TV)-specific expression values, the nonoverlapping pieces of each pri-miRNA transcript were quantified using HOMER (analyzeRepeats.pl with parameters -strand + -noadj -noCondensing -pc 3). The lengths of the quantified region and total read counts per sample were used to report normalized signal levels (RPKM). The contribution of each TSS (named TSSi) to the overall transcriptional activity in a given locus was determined by subtracting the signal level at the upstream element (named TSSi+1), based on the RPKM (reads per kilobase of transcript per million mapped reads) values: RPKMi=RPKMi−RPKM(i+1).

The differential expression of the primary miRNA transcripts between the different cell types was calculated using DESeq.

Super-enhancer locations were obtained from previous reports.[41,42] The distance between the super-enhancers' location and the mature miRNAs were calculated using the HOMER annotatePeaks algorithm using the -pdist option.

To detect the transcription factor enriched under the studied stimuli, we pooled the tag directories from each cell type separately under all stimuli. All transcribed regions were identified using HOMER v4.9 (http://homer.ucsd.edu/homer/[43]) with the command findPeaks.pl with the option -groseq. The expression of each transcript was further quantified using the HOMER command analyzeRepeats.pl for all the tag directories. The transcript start sites (±200 bp) of each transcript displaying a fold change above 1 between different conditions were further analyzed to detect the enriched transcription factor using CiiiDER.[44] To achieve this, the hg19 coordinates were converted to hg38 using the convertcoordinates.pl tool from HOMER and the fasta-sequences were extracted using BEDTools[40] (getfasta).

Analysis of miRNA Targets and Function

To study the biological effects of the expressed miRNAs, the MicroRNA Target Filter of Ingenuity Pathway Analysis (IPA, Qiagen Redwood City, www.qiagen.com/ingenuity) and miRWalk (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) were used. MicroRNA Target Filter uses experimentally determined microRNA targets using miRecords[45] and TarBase[46] alongside with manual curation from published literature. IPA's target filter was used to analyze the full lists of the expressed miRNAs with their expressional changes under proatherogenic stimuli for one cell type at a time. On the contrary, miRWalk has been shown to present better accuracy and precision than other available programs.[47]The data sets were coupled with their respective RNA-seq data sets and filtered to include those miRNA target genes that showed opposite expression trends from the miRNAs and thus could be under canonical posttranscriptional regulation. The addition of this correlation between miRNAs and their potential targets allows us to be more accurate in the miRNA target definition. We further filtered the targets to contain those most relevant to cardiovascular disease. Then, we compiled the target lists with their expression changes and entered them into IPA's Core Analysis, and finally continued to perform an IPA Comparison Analysis to bring all the data sets together into one analysis.

To study the function and interaction of a selected miRNA set, we used miRWalk[47,48] and compiled putative target lists for the miRNAs. We included targets, which were present in at least 3 of the 6 target prediction databases for 5′ UTR targets, or 4 of 12 target prediction databases for 3′ UTR and coding sequence targets. The list was further filtered to contain only those targets that were differentially expressed (RNA-seq/GRO-seq exhibiting a false discovery rate [FDR] <0.05) under proatherogenic stimuli compared with control conditions. These targets were uploaded to IPA for core analysis, and a subsequent comparison analysis to determine the canonical pathways and functions that were affected by the proatherogenic stimuli and the selected miRNAs.

RESULTS

Promoter Usage and Transcript Variant Expression of pri-miRNAs Is Cell-Type-Dependent

We first generated GRO-Seq data from HAECs, HUVECs, HASMCs, and CD14+ macrophages to compare the nascent pri-miRNA transcription profiles between the 3 cell types. Here, the cell-type specificity could arise from either pri-miRNA (1) being expressed only in one of the studied cell types or (2) exhibiting alternative usage of TSS and thus resulting in pri-miRNAs of different lengths. To evaluate these options, we used our previously described pri-miRNA transcript variant annotation tool.[21] Altogether, we identified 781 pri-miRNAs expressed in at least one condition in all replicates of a single cell type (RPKM >0.5). Despite most pri-miRNAs being expressed in all cell types, the majority of them (681/781, 87%) were differentially expressed (FDR <0.05) between the cell types under all conditions and for 453 (67%) pri-miRNAs the difference was over 10-fold in at least one pair-wise comparison of cell types (Figure 1A and 1B). Of these, 24% (110/453) exhibited alternative TSS usage as exemplified by a miR-23b/27b/24-1 cluster (Figure 1C and 1D). We did not observe clear stimuli-specific patterns in TSS usage and transcript variant expressions, which suggests that the expression of pri-miRNAs is mainly regulated in a cell-type-specific manner (Figure 1C, Figure II in the Data Supplement). Altogether, our data provides evidence that pri-miRNA transcription and TSS usage is highly cell-type specific and minimally affected by the proatherogenic stimulus.

Although the majority of pri-miRNAs were differentially expressed over 10-fold, only 84 pri-miRNAs were exclusively expressed (RPKM <0.5 in the 3 other cell types) in a given cell type (Figure 1E). These are exemplified by previously described MIR143, MIR145 (which are essential for HASMC functions),[50–53] MIR126, endothelial cell-specific miRNA regulating vascular inflammation[54] and MIR342, that promotes macrophage-driven inflammation.[55,56] However, high similarity in the pri-miRNA expression was seen between HUVECs and HAECs, as only 6 pri-miRNAs were differentially expressed including MIR339, MIR3194, MIR3938, MIR10B, and MIR196B for HUVEC and MIR320E for HAEC. Altogether, this analysis provides a resource for investigation of pri-miRNA transcription in atherosclerosis-associated cell types.

Majority of Expressed miRNAs Are Shared Between Cell Types and Dominated by the Expression of Few miRNAs

Next, we set out to compare the cell-type-specificity of nascent pri-miRNAs to the mature miRNA. To this end, we analyzed the miRNA profiles of HUVECs, HAECs, HASMCs, and CD14+ macrophages in response to 7 hours proatherogenic stimuli (oxPAPC, hypoxia, and oxPAPC under hypoxia) in matching samples. Similarly to pri-miRNAs, a large fraction (45%) of the ≈500 miRNAs were expressed in all the cell types, irrespective of conditions (Figure 2A, Figure III and Table II in the Data Supplement). However, a small subgroup of nonuniformly expressed miRNAs (from Figure IIIA and IIIB in the Data Supplement) were able to distinguish the cell types from each other (Figure 2B). Among these miRNAs, only 30% to 50% were identified as cell-type specific at the level of pri-miRNA transcription (Figure IVA in the Data Supplement) and the expression levels exhibited poor correlation (Figure 2C). This

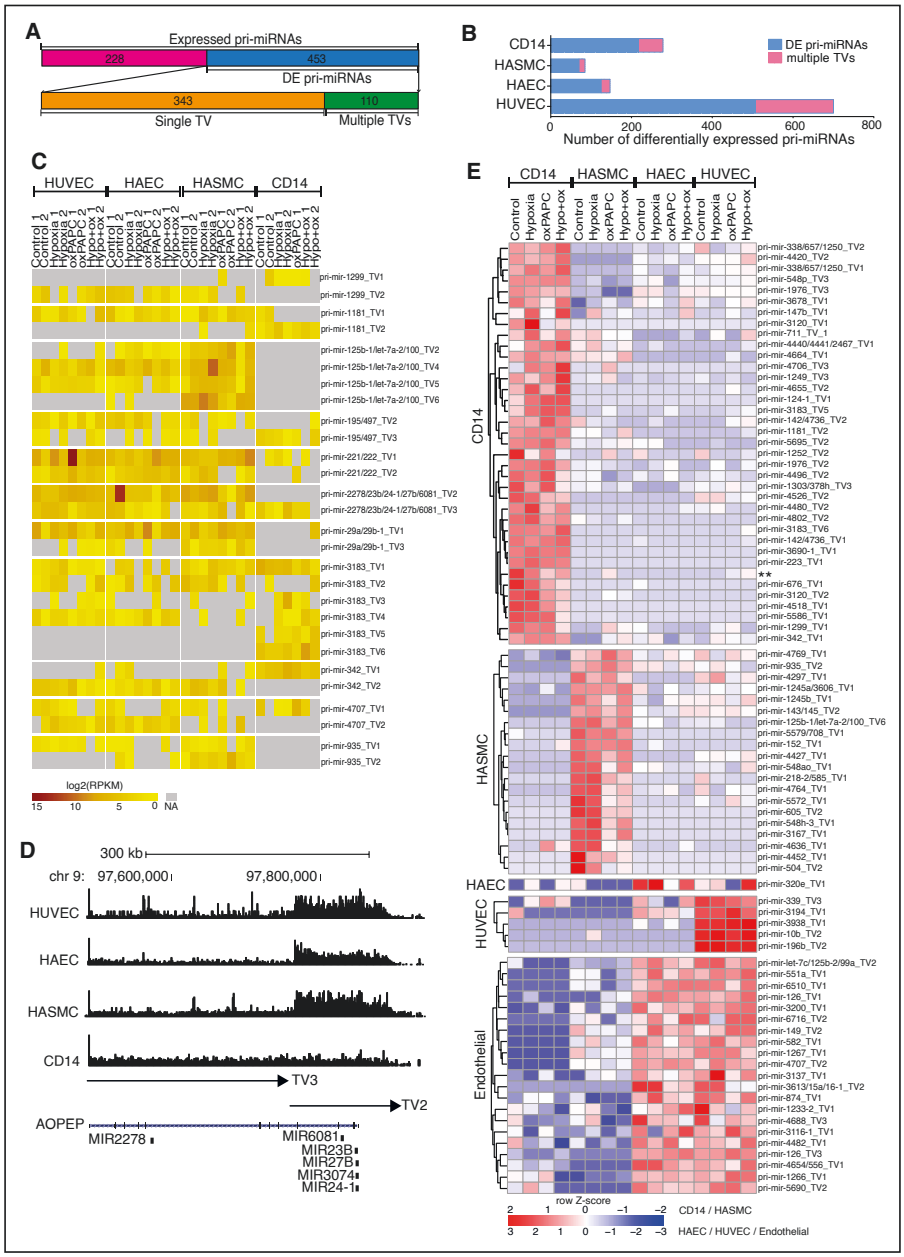

Figure 1. Promoter usage and transcript variant expression of pri-miRNAs is cell-type-dependent.
A, The numbers of pri-miRNAs expressed (false discovery rate [FDR] <0.05) in all cell types and the fraction of differentially expressed pri-miRNAs (FDR <0.05 and fold change >10 comparing 2 cell types). **B**, Fraction of DE (FDR <0.05) pri-miRNAs exhibiting multiple transcript variants (TVs). **C**, Heatmap showing transcript variant (TV) expression in log2 (RPKM [reads per kilobase of transcript per million mapped reads]) in different cell types and conditions. The contribution of each transcriptional start site (TSS) to the overall transcriptional activity in a given locus was determined by subtracting the signal level at the upstream element. **D**, An *MIR23B* cluster is shown as an example of cell-type-specific promoter usage and transcript variant expression, as 3 of the cell types (human umbilical vein endothelial cell [HUVEC], human aortic endothelial cell [HAEC], and human aortic smooth muscle cell [HASMC]) express 2 TVs, namely TV2 and TV3, and macrophages (CD14) only one of the TVs (TV3). University of California Santa Cruz (UCSC) genome browser shot demonstrating global run-on sequencing (GRO-seq) signal around the miRNA locus.[49] **E**, Row normalized expression (Row z-score) of cell-type specific pri-miRNAs separated according to the cell-type of origin. The rows are hierarchically clustered using the Euclidian distance and complete linkage clustering. **pri-miR-3179-2/3180-2/3670-2/6511a-2/6511a-3/3180-3/6511a-4_TV3. AOPEP indicates aminopeptidase O.

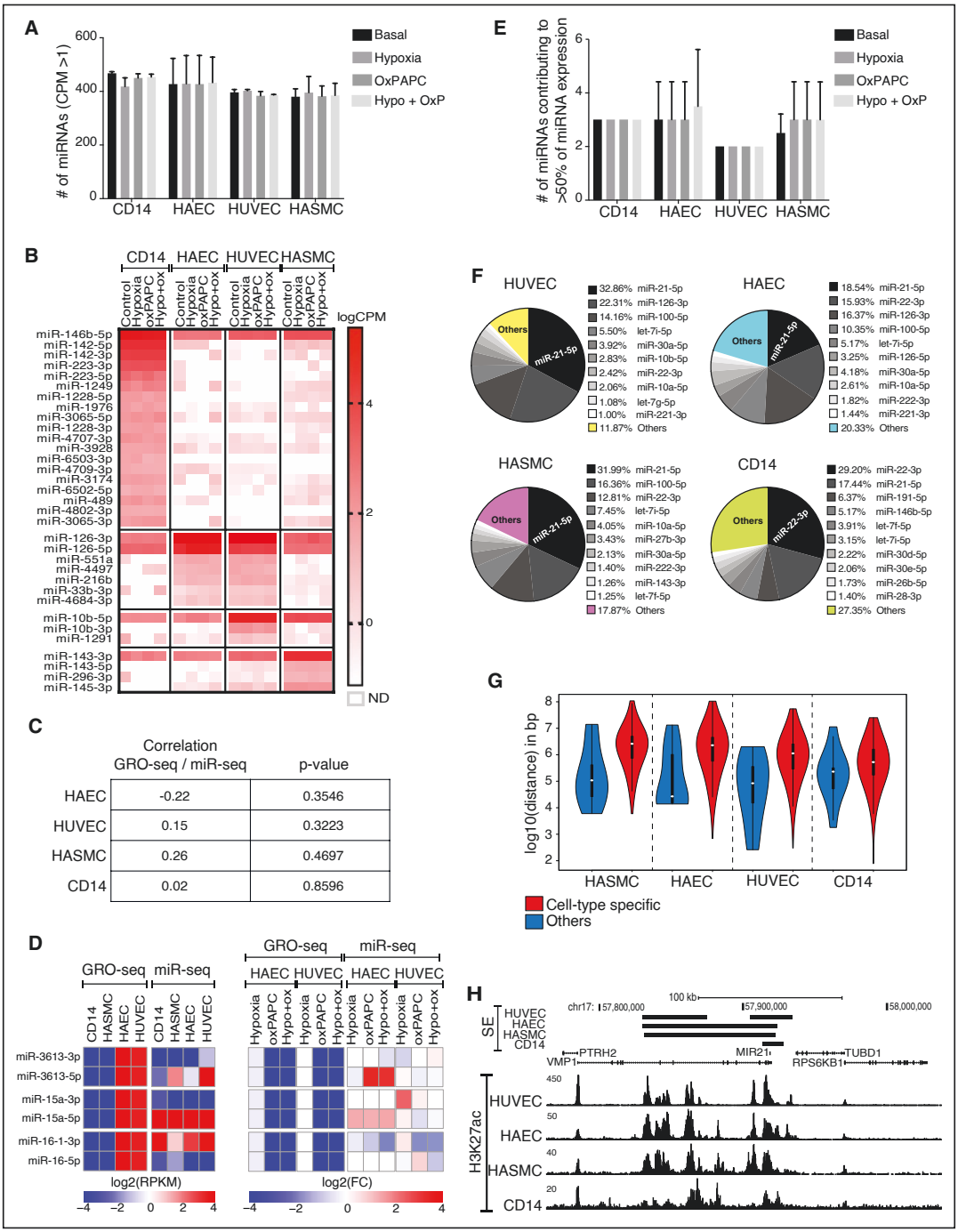

Figure 2. Majority of expressed miRNAs are shared between cell types and dominated by the expression of few miRNAs.
A, Basally expressed miRNAs. The number of expressed miRNAs (counts per million reads mapped [CPM] >1 and present in all samples of a condition) in each cell type in basal conditions and under proatherogenic stimuli (hypoxia, oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine [oxPAPC], and hypoxia+oxPAPC) is plotted (mean, SD, n=2 for each condition). **B**, Heatmap for selected cell-type-specific miRNAs. Values are plotted as logCPM. ND indicates not detected. **C**, Spearman correlation between the expression of cell-type specific pri-miRNAs (*Continued*)

Arterioscler Thromb Vasc Biol. 2021;41:2149–2167. DOI: 10.1161/ATVBAHA.121.315579

relation was further supported by the observation that only 14% (12/84) of the 84 cell-type exclusive pri-miRNAs (from Figure 1E) were expressed in a cell-type specific manner on a mature miRNA level (Figure IVB in the Data Supplement). Altogether, our data provides evidence that miRNA expression is strongly controlled at the level of posttranscriptional regulation. This was clearly demonstrated at the level of miRNA clusters such as miRNAs arising from *MIR3613* host gene, namely miR-15a-3p, miR-15a-5p, miR-16-1-3p, miR-16-5p, miR-3613-5p, and miR-3613-3p, which are transcribed as one pri-miRNA, but results in several mature miRNAs with very different expression values (Figure 2D).

The Functional Annotation of the Mammalian Genome project recently published[11] that miRNA expression levels differ highly and are extremely skewed, with about 5 miRNAs being responsible of half of the total miRNA expression in a given sample. In line with this, 2 to 5 miRNAs were confirmed to be the source of roughly 50% of the total miRNA expression in the cells (Figure 2E). The number was the lowest in HUVECs where only 2 miRNAs, namely miR-126-3p and miR-21-5p, contributed to 56% of the total miRNA expression in basal conditions and under stimuli. Overall, the top 10 miRNAs expressed contributed to 73% to 88% of the total miRNA expression and were largely invariant to stimulus (Figure 2F, Figure VA and VI in the Data Supplement). Thus, a surprisingly small set of miRNAs, many of which are ubiquitously expressed, account for significant differences in the miRNA profiles between different cell types and states. A comparison of the top 10 miRNAs arising from different cell types revealed the most similar profiles in the endothelial subtypes, HUVECs and HAECs, and the least similarity between the macrophages (CD14) and the rest of the cell types (HUVECs, HAECs, and HASMC; Figure 2F, Figures VB and VI in the Data Supplement). Interestingly, we also observed that the top 10 miRNAs were associated with closer proximity to super-enhancers, suggesting a potential mechanism accounting for their high expression level (Figure 2G). To this end, miR-21 had a super-enhancer detected in all the cell types analyzed here (Figure 2H).

High Similarity of Pri-miRNA Expression Changes in Response to Single or Combined Treatment

Next, we investigated the effect of different stimuli on the expression of pri-miRNAs. Altogether, 569, 430, and 515 pri-miRNAs were differentially expressed (GRO-seq, FDR <0.05) upon hypoxia, oxPAPC, or combined treatment, respectively. Altogether a larger fraction of DE pri-miRNA was shared between the cell types (shared pri-miRNAs—hypoxia: 27.5%, OxPAPC: 3.5%, HypoOxP: 20.7% [Figure 3A and Figure VIIA in the Data Supplement]), compared with nascent mRNA expressions where the larger fraction was cell-type specific (shared nascent mRNAs—hypoxia: 16.02%, OxPAPC: 1.37%, HypoOxP: 12.4% [Figure VIIC in the Data Supplement]). However, for both pri-miRNAs and mRNAs, the single stimulus responsive transcripts were also differentially expressed by the other single stimulus and by the hypoxia+oxPAPC combination (Figure 3A, Figures VIIB, VIID, VIIIA, and VIIIB in the Data Supplement). This suggests that transcriptional changes in the hypoxia+oxPAPC group are provoked by either oxPAPC or hypoxia, or that all the treatments have a similar direction of effect. To test these options, we extracted stimuli-specific signatures (Figure 3A and 3B) by assigning the differentially expressed pri-miRNAs (GRO-seq, FDR <0.05) in each data set into 7 groups: (1–3) oxPAPC, hypoxia, and hypoxia+oxPAPC only, which contained pri-miRNAs that were upregulated in one group and downregulated in the others (or vice versa); (4–6) combinations of the 3 (oxPAPC and hypoxia/oxPAPC and hypoxia+oxPAPC/hypoxia and hypoxia+oxPAPC), which contained pri-miRNAs that were upregulated in 2 of the groups and downregulated in the third (or vice versa); and (7) pri-miRNAs similarly expressed under all 3 stimuli (upregulated/downregulated in all). In a given data set, one pri-miRNA could only be assigned to one group, for example, in oxPAPC-treated cells, pri-miRNAs belonging to the oxPAPC only group were extracted first, then pri-miRNAs belonging to combinations and all categories. Additionally, pri-miRNA was required to be significantly differentially expressed in the treatment group that was under investigation and only the direction of expression (up/no change/down) was noted in the other treatment groups. The results showed

Figure 2 Continued. and the corresponding expression of mature miRNA in normoxia. Paired Student *t* test (2-tailed) was used to calculate the correlation's *P* value. **D**, Endothelial cell specific *MIR3613* host gene cluster expression detected using global run-on sequencing (GRO-seq) and miR-seq. Cluster miRNAs are transcribed as one pri-miRNA but result in several mature miRNAs with different expression values. Average expression of all treatments in log2 (RPKM [reads per kilobase of transcript per million mapped reads]; **left**) and log2 (fold change [FC]) comparing different stimuli to the control (**right**) are shown. **E**, The number of miRNAs that contribute to at least 50% of the total miRNA expression in each cell type/condition (mean, SD, n=2). **F**, Percentage of top 10 miRNAs expressed in each cell type. The values represent averages of all treatments and basal conditions for each cell type. **G**, Violin plot showing the distance in log10 (base pair) between the mature microRNA location and the closest super-enhancer's peak. Cell-type-specific mature miRNAs are the top 10 miRNAs in Figure 3F, while Others are all the microRNAs considered expressed (>1 CPM in at least 2 samples. The violin plots were created using the vioplot package [version 0.3.2] in R [version 3.5.2]). **H**, University of California Santa Cruz (UCSC) genome browser shot images of *MIR21*. Normalized tag counts are shown for H3K27ac ChIP-Seq tracks. Black bars represent the super-enhancer position for each cell type. HAEC indicates human aortic endothelial cell; HASMC, human aortic smooth muscle cell; HUVEC, human umbilical vein endothelial cell; PTRH2, peptidyl-TRNA hydrolase 2; RPS6KB1, ribosomal protein S6 kinase B1; TUBD1, tubulin delta 1; and VMP1, vacuole membrane protein 1.

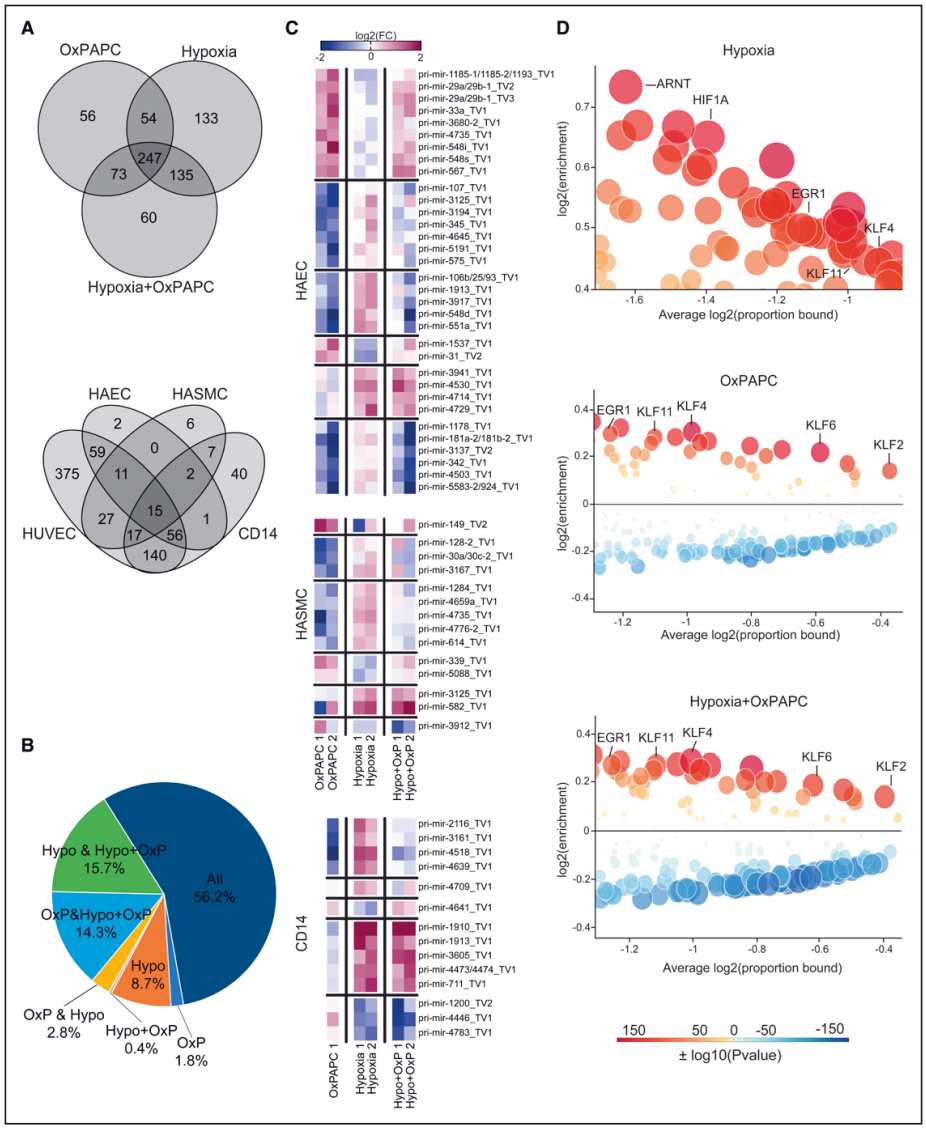

Figure 3. High similarity of primary microRNA (pri-miRNA) expression changes in response to single or combined treatment. **A**, Overlap of differentially expressed pri-miRNAs under all stimuli (**top**) and for all cell types (**bottom**) studied. **B**, Prevalence of the assigned miRNA groups is shown. Differentially expressed pri-miRNAs (global run-on sequencing [GRO-seq], FDR <0.05) in each data set have been assigned to one of the 7 groups. Each miRNA has been assigned to a group and each miRNA can only be present in one of the groups. **C**, Log2FC of the pri-miRNA expression under the studied stimuli compared with the control. Heatmaps were generated using Graphpad Prism 8. **D**, Enrichment analysis showing a selection of over-represented and under-represented motifs at the transcriptional start site (TSS) of transcripts regulated at least 2-fold by each stimulus. The plots were generated using CiiiDER.[44] ARNT indicates aryl hydrocarbon receptor nuclear translocator; EGR1, early growth response 1; HAEC, human aortic endothelial cell; HASMC, human aortic smooth muscle cell; HIF1A, hypoxia-inducible factor 1 subunit alpha; HUVEC, human umbilical vein endothelial cell; KLF, Krüppel-like factor; oxPAPC, oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine; and TV, transcript variant.

that the majority of the pri-miRNAs were expressed similarly under all stimuli, and only ≈11% of the pri-miRNAs were found to respond stimuli specifically (Figure 3B and 3C, Figure VIIIC in the Data Supplement). Taken together, our results suggest that hypoxia, oxPAPC, and combined treatment exhibit surprisingly similar directions of effect on pri-miRNA expressions and thus likely involve similar regulatory mechanisms.

To further analyze the commonalities in the transcriptional mechanisms due to hypoxia and oxPAPC, we

analyzed the de novo motif enrichment at the TSS of regulated transcripts (fold change >2 in at least one comparison in at least one cell type) for each stimulus. We identified 679, 140, and 172 enriched motifs for hypoxia, oxPAPC and hypoxia+oxPAPC, respectively. On the contrary, 1329 and 361 motifs for hypoxia, oxPAPC, and hypoxia+oxPAPC, respectively, were depleted compared with the background. In line with the pri-miNA expression profiles, most of the enriched motifs contributing to the combined response were shared between hypoxia and oxPAPC stimuli separately (Figure 3D, Table III in the Data Supplement). Indeed, only 2 motifs were specific in hypoxia+oxPAPC, suggesting that most motifs were also enriched in the single stimulus responsive TSSs. Among the top enriched motifs in hypoxia+oxPAPC, we identified previously validated signal responsive TFs (transcription factors) such as ARNT (aryl hydrocarbon receptor nuclear translocator) mediating the hypoxia response[57] or Zfp148 (zinc-finger protein 148) and ERG-1 (early growth response 1) mediating the response to oxidative stress,[17,58] as well as many members of the KLF (Krüppel-like factor) family of TFs that have been shown to contribute to proatherogenic gene expression changes.[59] A comparison of the single stimuli demonstrated that only 1 motif was specific to oxPAPC, whereas hypoxia exhibited a larger set of stimulus-specific motifs. To provide further evidence of the involvement of the predicted TFs in the regulation of miRNAs, we used TransmiR v2.0.[60,61] that makes use of literature-curated TF–miRNA regulation data. This allowed confirmation of the KLFs, HIF1a (hypoxia-inducible factor 1 subunit alpha)/ARNT, and EGR1 in the regulation of miRNAs (Figure IX in the Data Supplement). Altogether, we present evidence that there is high similarity in the pri-miRNA responses to the proatherogenic stimuli between the cell types, which could be partly due to the sharing of many TFs that mediate the responses to hypoxia and oxidative stress.

MicroRNA Target Genes Are Associated With Atherosclerosis-Related Functions

Most studies comparing atherosclerosis-associated changes in miRNA expression profiles have been performed at the level of bulk tissue, leaving the cell of origin of the differential signals unclear. To address this knowledge gap and to analyze the relevance of our miRNAs in the disease context, we collected information of the differentially expressed miRNAs from all published studies conducted in human atherosclerotic plaques to date[62–66] and analyzed their miRNA expression level and stimulus response in each of the 3 cell types (Figure 4A). Importantly, a highly cell type-specific expression pattern of the mature miRNAs was observed as exemplified by macrophage-specific expression of miR-223-3/5p and endothelial-specific expression of miR-99a-5p, miR-126-3p, miR-146a-3p, and miR-196a/b-5p. Furthermore, >85% (45/52) of the pri-miRNAs were found differentially expressed (FDR <0.05) in at least one condition supporting the disease relevance of our findings. These findings suggest that several of the disease-relevant miRNAs respond to proatherogenic stimulus at transcriptional level and their expression can be assigned to one cell type predicting the potential cell type of action.

Although the mature miRNA profiles in different cell types or under proatherogenic stimuli did not show drastic differences (Figure III in the Data Supplement), the miRNA functions could differ as the target gene pools are not the same between the cell types, with about 5% of the mRNAs being exclusively expressed in one cell type or exhibiting over 8-fold difference in expression level (Figure XA and XB in the Data Supplement). To study the biological effects of the expressed miRNAs further in all cell types under proatherogenic stimuli, we used the IPA MicroRNA Target Filter that relies on experimentally validated and predicted mRNA targets. Inspection of the results of the Diseases and Functions section revealed several changes in the cellular functions that indicate the entering and maintenance of a proatherogenic state, such as increased endothelial activation, vascular permeability and cellular movement, and migration of the cells (Figure 4B). In addition to the prodisease changes, the overall results showed clear indications of changes that would, in the plaque environment, increase the vulnerability of the plaque and the susceptibility to adverse cardiac events, such as increased smooth muscle cell death and decreased proliferation, increased neovascularization, and increased platelet activation and thrombus formation.[67] Overall, there was a high similarity in the directionality of the effect (z-score) for the majority of the functions, suggesting that the majority of the effects mediated by the target mRNAs are concordant between the cell types. However, activation of the cell migration, vascularization, and inhibition of apoptosis was more predominant in endothelial cells compared with HASMCs and CD14+ macrophages. Cell-type-specificity was also evident from the target mRNA changes, as shown for the atherosclerosis signaling molecules in Figure XC in the Data Supplement. A schematic of the possible interactions of the different cell types and signaling molecules within the plaque environment is provided in Figure 4C. The full table of miRNAs and their predicted targets can be found in the Table IV in the Data Supplement.

miR-100-5p Promotes Atherogenesis-Associated Cellular Events by Regulating the HIPPO Pathway and Inflammation

To find miRNAs with significant effects on target gene expression and the genes most affected by the miRNA function, we filtered the data based on target numbers and by targeting miRNAs, respectively. The miRNAs with the highest target numbers in the data and genes

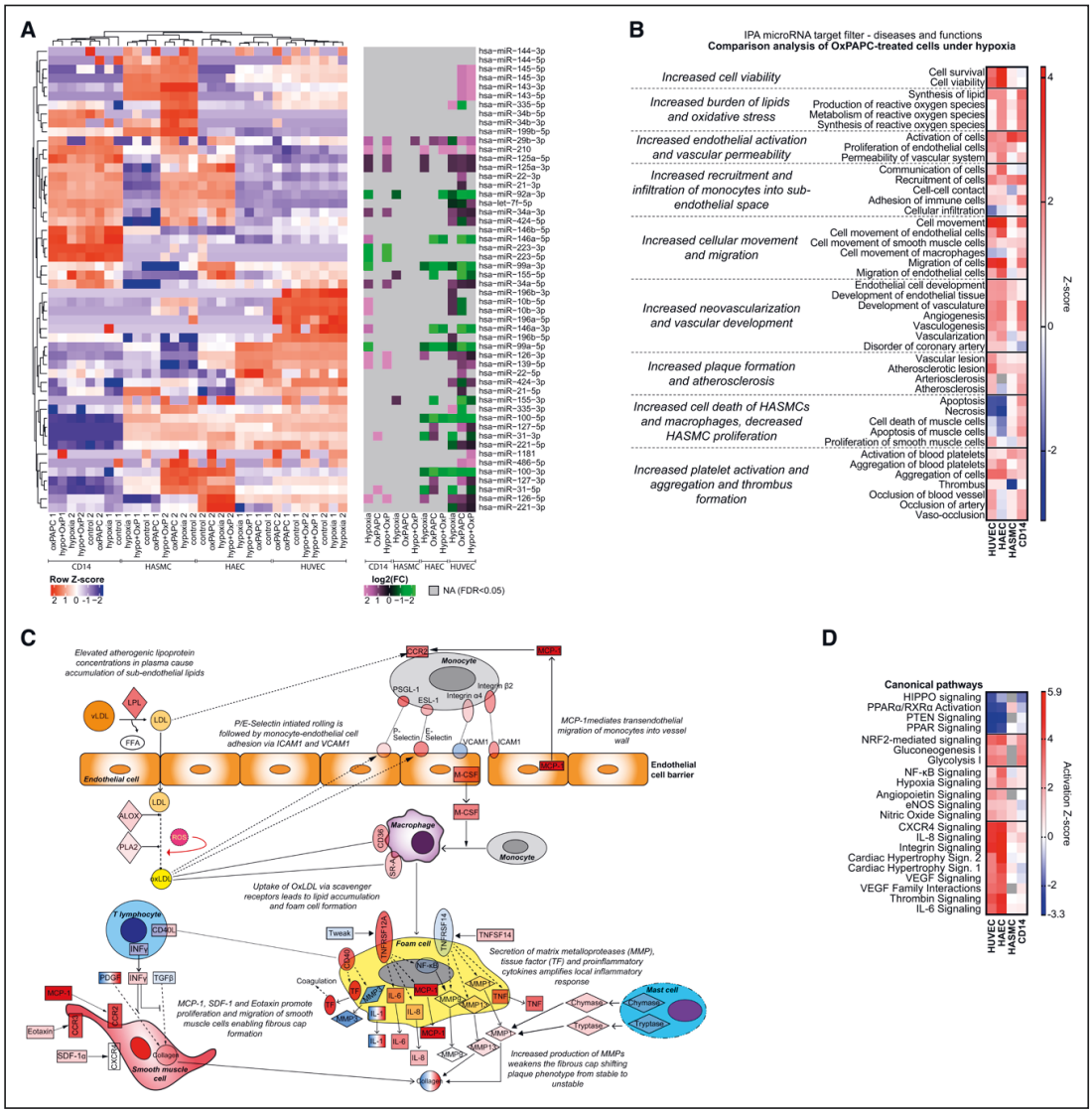

Figure 4. MicroRNA (miRNA) target genes are associated with atherosclerosis-related functions.
A, Heatmap of row-normalized expression of miRNAs found deregulated in human atherosclerotic plaques[62–66] (**left**) and the log2 fold change [FC] of those miRNAs compared with normoxia in global run-on sequencing (GRO-seq; **right**). **B**, Diseases and functions from IPA's Comparison Analysis for miRNA targets in oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine [oxPAPC]-treated cells under hypoxia. The heatmaps were generated using Graphpad Prism 8. **C**, Mechanistic summary of the proatherogenic functions of the miRNA target genes in the various cell types and their possible interaction with each other in the plaque environment. **D**, Selected canonical pathways from IPA's Comparison Analysis for targets of miR-21-5p, miR-22-3p, miR-100-5p, miR-34a-5p, and miR-92a-3p in oxPAPC-treated cells under hypoxia. Heatmaps were generated using Graphpad Prism 8. FDR indicates false discovery rate; HAEC, human aortic endothelial cell; HASMC, human aortic smooth muscle cell; HUVEC, human umbilical vein endothelial cell; IL, interleukin; INF, interferon; PDGF, platelet-derived growth factor; TF, transcription factor; and TGF, transforming growth factor.

with the highest number of miRNAs targeting them are summarized in Figure XI in the Data Supplement. For HUVECs, HAECs, and HASMCs, the miRNA lists mostly consist of miRNAs which are downregulated, and thus their targets are upregulated. For CD14+ the trend is the opposite. Overall, the miRNAs on the lists are very lowly expressed in the cells, and therefore, may not have strong biological effects, as argonaute

(AGO)-loading has been suggested to be dominated by the most abundant miRNAs. Indeed, by selecting the 5 of the most highly expressed miRNAs, namely miR-21-5p, miR-100-5p, miR-22-5p, miR-34a-5p, and miR-92a-3p, it was possible to largely recapitulate the predicted functional effects on the cell types (Figure 4D and Figure XD in the Data Supplement). Here, HIPPO signaling was found to be repressed by miRNA target prediction with the strongest effect detected in endothelial cells. A recent study suggests that YAP/TAZ activation in endothelial cells plays a causal role in the initiation and progression of atherosclerosis,[68] and the overexpression of miR-100-5p has been associated with vulnerable plaque phenotypes.[65,69,70] However, since the target mRNA predictions are solely based on bioinformatic predictions with limited accuracy, we sought to experimentally modulate miR-100-5p expression to validate the predicted miRNA-mediated effects on the YAP/TAZ pathway (Figure 5A, Figures XIIA and XIII in the Data Supplement). The results confirmed that in HUVECs the overexpression of miR-100-5p introduces a trend towards increased nuclear TAZ levels in proatherogenic conditions, whereas miR-100-5p silencing reduced the nuclear TAZ levels. The opposite was true for miR-100-5p silencing. However, in HASMCs, the trend was opposite, with the overexpression of miR-100-5p leading to a decrease in nuclear TAZ level and an increase in phosphorylated TAZ.

The evolutionary conserved HIPPO pathway is a key regulator of cell fate in response to biochemical and biophysical cues.[68,71,72] In addition to controlling organ development and growth as well as tissue homeostasis, the HIPPO pathway regulates metabolic processes at the cellular and organismal levels in both physiological processes and metabolic disease states, also in atherosclerosis. Silencing of the HIPPO signaling pathway leads to dephosphorylation and subsequent activation of the Yes-associated protein and Transcriptional Co-Activator With PDZ-Binding Motif (YAP and TAZ) further towards proproliferative and proinflammatory responses in cells, which promote atherogenesis. Thus, we next sought to investigate if the effect of miR-100-5p on the HIPPO pathway was dependent on stimulus or the cell type. To achieve this, we analyzed effects of miR-100-5p activation or inhibition on target mRNA expression in HUVECs and HASMCs under a control conditions and hypoxia+oxPAPC combination stimulus. Based on miRWalk-database,[47,48] the miR-100-5p was predicted to regulate the expression of several regulators of the HIPPO-YAP/TAZ cascade, including the pathway regulators *AJUBA*, *BMPR2*, *LATS2, PPP2R2D*, *PRKCZ*, and *SMAD7*. The results demonstrated highly similar regulation of the target mRNAs upon control conditions, whereas combination treatment revealed cell-type specific regulation of *LATS2* (HUVEC only), *AJUBA* (HASMC only), and *SMAD7* (HASMC only; Figure 5B). In addition, the effect of miR-100-5p on the *SMAD7* expression was only evident upon combined treatment in HASMCs. Interestingly, the effect of miR-100-5p on *LATS2* and *AJUBA* expression was opposite to what would be expected from the canonical miRNA regulation under the combination stimulus, suggesting that the effect was indirect. Altogether, this suggests that miR-100-5p displays both cell-type and treatment-specific control of the HIPPO pathway regulators.

The HIPPO pathway and miR-100-5p have both been shown to exhibit extensive crosstalk with pathways involved in proliferation, angiogenesis, and inflammation.[73–75] To provide an unbiased view of the cell-type specific effects of miR-100-5p, we performed RNA-Seq profiling in HUVECs and HASMCs transfected with miR-100-5p mimic under hypoxia+oxPAPC stimulus (Figure XIIB and Table V in the Data Supplement). To determine the biological pathways of the genes affected by the overexpression of miR-100-5p, we performed a Gene Set Enrichment Analysis of the genes ranked by FDR and the effect direction (Walk statistic rank; Table VI in the Data Supplement). The top biological pathway, epithelial to mesenchymal transition, was highly enriched among the upregulated genes in both cell types. However, the hallmark gene sets related to hypoxia, TNFα (tumor necrosis factor-alpha) signaling via NFκb (nuclear factor kappa B subunit 1), and IFNγ (interferon gamma) response exhibited positive enrichment in HASMCs, while a negative enrichment was seen in HUVECs (Figure 5C). This signifies that miR-100-5p could have an opposite effect on these 2 signaling pathways, with the upregulated genes in HASMCs and downregulated genes in HUVECs showing gene set enrichment. In contrast, only HUVECs demonstrated significant (FDR <0.1) positive enrichment for genes associated with proliferation (Mitotic spindle) and negative enrichment for mTORC (mechanistic target of rapamycin kinase) signaling and ROS (reactive oxygen species) pathway whereas in HASMCs genes related to TGF-β (transforming growth factor beta 1) signaling and apoptosis were positively enriched (Figure 5C and Figure XIIC in the Data Supplement). Our results suggest that complex networks of miRNA-mRNA interactions could exert opposing roles in different cell types of the same tissue. Importantly, we provide the first evidence of the participation miR-100-5p in the regulation of the HIPPO pathway in endothelial cells and its antagonistic role in inflammatory signaling in vascular cell types.

DISCUSSION

In this study, the mechanisms and signaling pathways associated with proatherogenic stimulus were studied

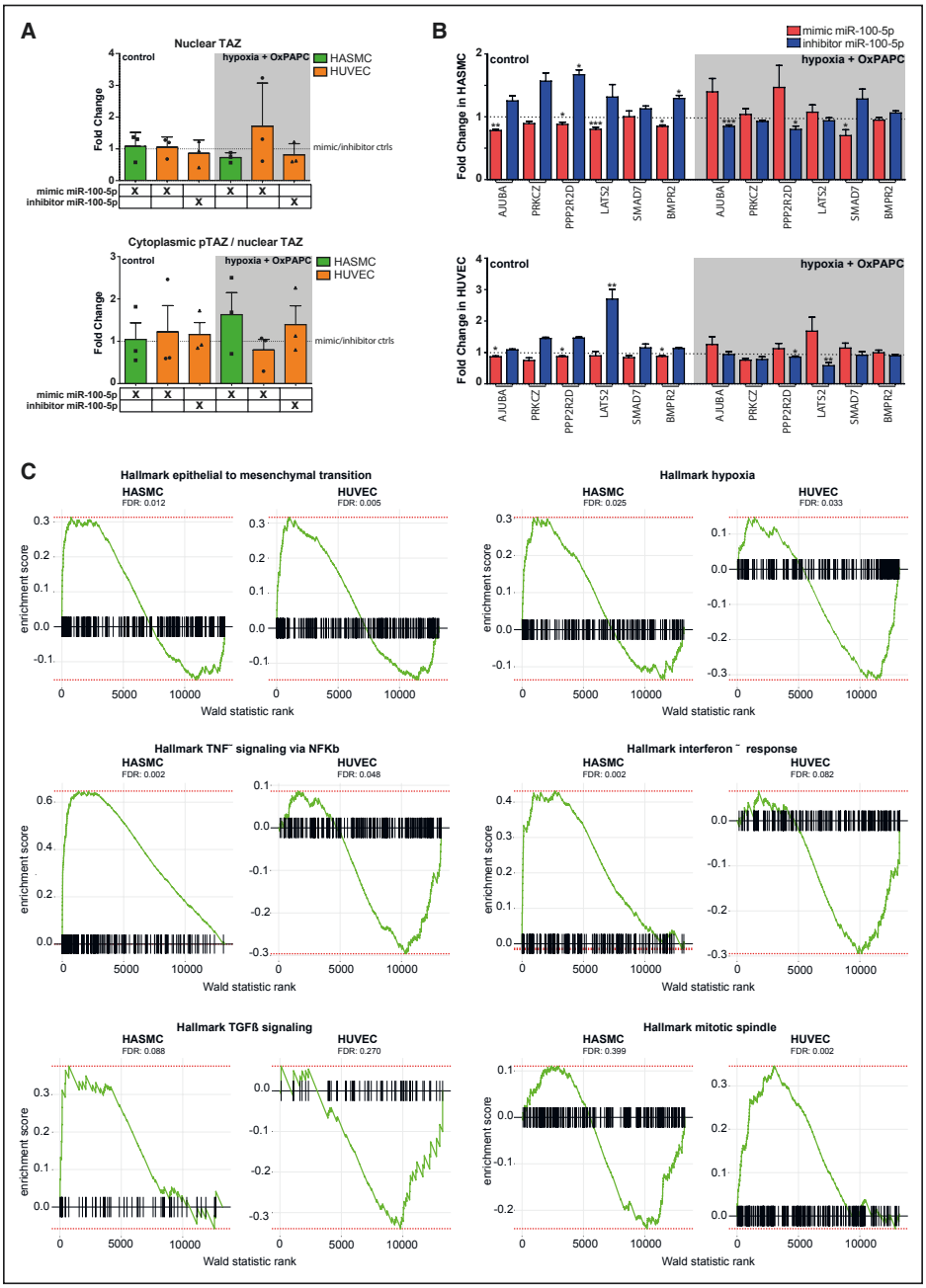

Figure 5. miR-100-5p promotes atherogenesis-associated cellular events by regulating the HIPPO pathway and inflammation. **A**, Western blot quantitation for nuclear TAZ normalized to Histone H3 (human umbilical vein endothelial cell [HUVEC]) or total protein (human aortic smooth muscle cell [HASMC]), and for cytoplasmic phosphorylated TAZ (pTAZ) and nuclear TAZ ratio from miR-100-5p inhibitor and mimic transfected cells. Cytoplasmic pTAZ was normalized to β-actin (HUVEC) or total protein (HASMC). All results are presented relative to the respective control values with the SD. n=3. **B**, Gene expression in HASMC (**top**) and HUVEC (**bottom**) samples transfected with miR-100-5p mimic (red) or inhibitor (blue) under normoxia or hypoxia+oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (OxPAPC) relative to respective controls. Data was checked for normal distribution before performing statistical tests. Paired Student *t* test (2-tailed) was used for data that followed normal distribution and equal variance. Otherwise, nonparametric Mann-Whitney test was used (mean±SD, n=6, **P*≤0.05; ***P*≤0.01; and ****P*≤0.001). **C**, GSEA analysis of selected hallmark gene sets after ranking the sequencing results of miR-100-5p overexpression using Wald statistics in HASMCs and HUVECs. FDR indicates false discovery rate; TAZ, transcriptional co-activator with PDZ-binding motif; TGFβ, transforming growth factor beta; and TNFα, tumor necrosis factor-alpha.

in disease-associated cell types by obtaining and integrating different levels of next-generation sequencing data, with a special focus on the miRNA expression and function under proatherogenic stimuli. First, we analyzed the pri-miRNA expression levels and TSS usage in HAECs, HUVECs, HASMCs, and CD14+ macrophages. In line with several previous studies,[21,76,77] our results provide strong evidence that the transcription of pri-miRNA and the TSS usage is largely cell-type specific. In addition, we demonstrate that the TSS usage is poorly affected by stimuli, further suggesting that the TSS activation is performed through cell-type specific regulatory elements that largely preexist before the stimulus.[78]

In our study, we also demonstrate that the correlation between pri-miRNA and mature miRNA expression was low for the cell types and stimulus, suggesting that miRNA expression is further regulated in a posttranscriptional manner. This is in line with a recent study based on metabolic labeling of nascent RNAs demonstrating that many pri-miRNAs indeed fail to generate mature miRNAs.[79] Likely reason for this is the extensive regulation of miRNA biogenesis that influence miRNA processing and turnover. Interestingly, previous studies have shown miRNA processing to play a stronger role over expression in determining the level of mature miRNAs. The microprocessor complex transforms pri-miRNA into pre-miRNA and the efficiency seems to be determined by motifs within the pri-miRNA (such as the GC dinucleotide motif within the miR-100 pri-miRNA), in addition to secondary structural features, as well as microprocessor cofactors.[80] Moreover, accessory proteins can bind and regulate the pri-miRNA or the microprocessor complex, and other regulators such as noncoding RNAs are also involved in the regulation.[81] Alternatively, mature miRNA levels can also be regulated during the nuclear export and at the level of cytoplasmatic processing,[80] nuclear import, and subcellular localization[5,82,83] and by the availability of the proteins participating in their biogenesis and stability such as DICER and AGO.[84] However, we chose 1-hour difference between GRO-Seq and miRNA-Seq based on previous studies showing that mRNA level temporally lag behind the corresponding changes in transcription rates by ≈15 to 30 minutes[85] thus allowing enough time for processing to occur. Still, we cannot exclude the possibility that the short time difference chosen partly contributes to the poor correlation observed between pri-miRNA and miRNA levels. Our analysis also demonstrates that the highest expressed miRNAs are found close to super-enhancers, which could contribute to the higher expression levels. Supporting this, a recent report has suggested that super-enhancers enable nascent pri-miRNA transcription, and facilitate Drosha/DGCR8 recruitment and pri-miRNA processing to boost cell-specific miRNA production.[86] Moreover, miRNA decay constitutes another mechanism by which the levels or miRNA are regulated. A recent study based on a pulse-chase approach on metabolic RNA labeling enabled the study of the heterogeneity of miRNAs half-lives. miRNA decay is achieved by modifying the miRNA ends, either by adding or trimming the nucleotides, as well as through interaction with the target mRNAs. Interestingly, miR-100 was classified as a fast decaying miRNA, which would make it possible to act in specific cellular responses that require a rapid change in the level of miRNA.[87] Since microprocessor regulation and miRNA decay are affected by physiological processes, future studies focused on the pri-miRNA regulation in atherogenesis are needed.

Our analysis identified several cell-type-selective miRNAs such as miR-142 and mir-223 for macrophages, miR-126-3p and miR-126-5p for vascular endothelial cells, and miR-143 and miR-145 for vascular smooth muscle cells, which have previously been shown to regulate essential functions and differentiation of these cell types.[51,88–91] Still, the majority of the miRNA expression was dominated by a few miRNAs that were largely shared between the cell types. This is in line with previous studies demonstrating that on average 5 miRNAs contribute to half of the total miRNA expression in a given sample.[11] Given that the most abundant miRNAs have been suggested to dominate posttranscriptional target gene regulation by AGO proteins, these miRNAs may also play critical roles in disease pathogenesis.[92,93] Indeed, miR-21 and miR-22 have been shown to be increased in several pathologies, such as cancer and cardiac hypertrophy, respectively.[94,95]

To our knowledge, this is the first study to characterize the combined response to hypoxia and oxidized lipids in the main cell types representative of atherosclerosis. To our surprise, most of the pri-miRNAs and the direction of regulation were the same for the single and combined stimulus. These results suggest that hypoxia, oxPAPC, and combined treatment have a similar direction of effect on pri-miRNA regulation in a given cell type and are, thus, likely to involve similar transcriptional regulatory mechanisms. By analyzing the motifs around the TSSs of those pri-miRNAs under different stimuli, we were able to identify stimulus-specific motifs and show that the combination of stimuli is solely the result of the interaction between the 2 stimuli-specific responses. Among the top enriched motifs, ARNT, also known as HIF1-β (hypoxia-inducible factor 1, beta subunit), participates in the hypoxia response by binding to HIF1α. Although it is extensively known that HIF1-β is constitutively expressed, some studies have revealed that it can be upregulated by hypoxia in a cell-type specific manner.[57] In addition, ARNT has been shown to be involved in the oxidative stress response through the activation of NRF2 (nuclear factor erythroid 2-related factor 2)[96] and recent studies have shown significant crosstalk

between the NRF2 and HIF1α signaling pathways.[97–99] Another enriched motif, the Zfp148 transcription factor, has been demonstrated to promote cell proliferation under oxidative stress conditions and its deficiency confers protection against atherosclerosis in vivo.[58] Moreover, oxPAPC has also been shown to induce an atherogenic EGR-1 expression in vivo, contributing to an atherosclerosis progression.[17] Under combined hypoxia and oxPAPC, several members of the KLF family were also among the top enriched motifs. Particularly, KLF2, 4, 6, and 11 have been shown to participate in different biological functions involved in cardiovascular diseases, such as regulation of inflammation, angiogenesis, and thrombosis in endothelial and smooth muscle cells.[59,100] Still, direct experimental validation of the transcription factors binding near the miRNA TSS are still needed to validate our findings.

By analyzing the target genes of the miRNAs, we identified the HIPPO pathway as one of the main targets of the 5 most highly expressed miRNAs. To confirm this prediction, we silenced and overexpressed miR-100-5p in HUVECs and HASMCs. Our data demonstrates that miR-100-5p exerts a highly cell and stimulus-specific regulation of the HIPPO pathway. This was exemplified by the nuclear localization of TAZ upon miR-100-5p overexpression in HUVECs but not in HASMCs and the HASMC-specific regulation of *SMAD7* (mothers against decapentaplegic homolog 7) under combination treatment. Recent evidence positions SMAD7 as a central coordinator of crosstalk between HIPPO- and TGF-β signaling[101,102] where SMAD7 has been shown to suppress the TGF-β signaling. This provides one potential mechanism that could explain the cell type-specific upregulation of TGF-β pathway hallmark genes in HASMCs that warrants further research.

Importantly, a recent study demonstrated extensive crosstalk between mTORC and HIPPO pathways by showing that when the HIPPO pathway is active, mTORC1 signaling is turned off and vice versa.[102] In line with the cell-type-specific regulation of the HIPPO pathway, the genes related to mTORC pathway were only enriched among the miR-100 downregulated genes in HUVECs. Repression of mTORC signaling has been further mechanistically linked to an anti-inflammatory reaction to atherosclerotic stimuli in HUVECs under miR-100 overexpression.[70] To this end, we also demonstrate a significant representation of TNFα-, IFNβ-, and hypoxia-pathway mediators among the downregulated genes in HUVECs whereas the opposite was seen in HASMCs. Our findings could also provide a partial explanation to the different functional effects previously reported for miR-100, where inhibition of miR-100 had a significant stimulatory effect on HASMC migration whereas no effect was seen in HUVECs.[75] In summary, our study identifies miR-100-5p as a prominent player mediating the cell-type specific regulation of inflammation and hypoxia associated genes. Still, we acknowledge that our work is limited to in vitro experiments, and we cannot directly infer mechanisms of atherogenesis from our results. Future analysis and perturbation of cell-type specific miRNA expression in the tissue context is hoped to address these limitations.

Altogether, our work reveals a greater complexity in miRNA regulation than previously known and provides a resource for investigations on cell-type specific differences in miRNA transcription in the vascular wall. This information could be used to further characterize the atherosclerosis relevant regulatory networks and serve as the basis for future development of cell-targeted therapeutics.[104,105]

ARTICLE INFORMATION

Received October 13, 2020; accepted April 28, 2021.

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Acknowledgments

We would like to thank the EMBL Sequencing Service GeneCore Sequencing Facility (EMBL, http://www.genecore.embl.de) for the Next-Generation Sequencing (NGS) library sequencing and the University of Eastern Finland (UEF) Bioinformatics Center for server infrastructure. Sequencing data from this study has been submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus under accession number GSE136813 and GSE154427. The public data sets for CHiP-seq can be found under accession number GSE44288[41] and GSE52642[42] and the miRNA profiling of human umbilical vein endothelial cells under normoxia and hypoxia (24 hours) can be found under accession number GSE26497.[106] The super-enhancers were obtained from GSE51522[107] and GSE52642.[42]

Sources of Funding

This work was supported by the Academy of Finland (Grants Nos. 287478, 294073, and 319324 to M.U. Kaikkonen, 321535 and 328835 to J.P Laakonen), the Emil Aaltonen Foundation (to S. Linna-Kuosmanen), the Finnish Foundation for Cardiovascular Research (to S. Linna-Kuosmanen, P.R. Moreau and M.U. Kaikkonen), the Maud Kuistila Memorial Foundation (to S. Linna-Kuosmanen and P.R. Moreau), the Orion Research Foundation (to S. Linna-Kuosmanen), the Jane and Aatos Erkko Foundation (to M.U. Kaikkonen), the Sigrid Jusélius Foundation (to S. Linna-Kuosmanen and M.U. Kaikkonen) and Ella and Georg Ehrnrooth Foundation (to J.P. Laakkonen). This research was supported by the Doctoral Programme in Molecular Medicine (DPMM). This project was partly funded by the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant No. 802825 to M.U. Kaikkonen, 931683 to S. Ylä-Herttuala). Funding for open access is provided by Academy of Finland.

Disclosures

None.

Supplemental Materials

Online Figures I–XII
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II

Transcriptional Profiling of Hypoxia-Regulated Non-coding RNAs in Human Primary Endothelial Cells

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Frontiers in cardiovascular medicine 5:159, 2018

* and **denotes authors with equal contribution

frontiers in Cardiovascular Medicine

ORIGINAL RESEARCH
published: 05 November 2018
doi: 10.3389/fcvm.2018.00159

Transcriptional Profiling of Hypoxia-Regulated Non-coding RNAs in Human Primary Endothelial Cells

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OPEN ACCESS

Edited by:
Johannes A. Schmid,
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Reviewed by:
Ulrike Resch,
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Specialty section:
This article was submitted to Atherosclerosis and Vascular Medicine, a section of the journal Frontiers in Cardiovascular Medicine

Received: 15 August 2018
Accepted: 16 October 2018
Published: 05 November 2018

Citation:
Moreau PR, Örd T, Downes NL, Niskanen H, Bouvy-Liivrand M, Aavik E, Ylä-Herttuala S and Kaikkonen MU (2018) Transcriptional Profiling of Hypoxia-Regulated Non-coding RNAs in Human Primary Endothelial Cells. Front. Cardiovasc. Med. 5:159. doi: 10.3389/fcvm.2018.00159

Hypoxia occurs in human atherosclerotic lesions and has multiple adverse effects on endothelial cell metabolism. Recently, key roles of long non-coding RNAs (lncRNAs) in the development of atherosclerosis have begun to emerge. In this study, we investigate the lncRNA profiles of human umbilical vein endothelial cells subjected to hypoxia using global run-on sequencing (GRO-Seq). We demonstrate that hypoxia regulates the nascent transcription of ∼1800 lncRNAs. Interestingly, we uncover evidence that promoter-associated lncRNAs are more likely to be induced by hypoxia compared to enhancer-associated lncRNAs, which exhibit an equal distribution of up- and downregulated transcripts. We also demonstrate that hypoxia leads to a significant induction in the activity of super-enhancers next to transcription factors and other genes implicated in angiogenesis, cell survival and adhesion, whereas super-enhancers near several negative regulators of angiogenesis were repressed. Despite the majority of lncRNAs exhibiting low detection in RNA-Seq, a subset of lncRNAs were expressed at comparable levels to mRNAs. Among these, MALAT1, HYMAI, LOC730101, KIAA1656, and LOC339803 were found differentially expressed in human atherosclerotic lesions, compared to normal vascular tissue, and may thus serve as potential biomarkers for lesion hypoxia.

Keywords: long non-coding RNA, atherosclerosis, hypoxia, endothelial cell, super-enhancer, GRO-Seq

INTRODUCTION

Recent transcriptomic analyses have established that up to 90% of the eukaryotic genome is transcribed (1). Only 2% of these transcripts encode for proteins, while the vast majority is transcribed as non-coding RNAs (ncRNAs). An increasing number of reports have discovered functional and structural roles for ncRNAs (e.g., microRNAs, small nucleolar RNAs, and small nucleolar RNAs), but despite this, the majority of them remain uncharacterized. The largest group of ncRNAs are called long ncRNAs (lncRNAs), which are defined as non-coding transcripts >200 nucleotides in length (2). LncRNAs can be further divided into promoter-associated lncRNAs and enhancer-associated lncRNAs (also called enhancer RNAs) based on epigenomic classification (3). Promoter-associated lncRNAs, like protein-coding mRNAs, are relatively stable, often spliced

and polyadenylated, whereas enhancer RNAs (eRNAs) tend to lack these modifications and are generally unstable (4). Interestingly, lncRNA expression is exquisitely cell type-specific and is often perturbed in disease states (5, 6), suggesting functions in development, homeostasis and maintenance of cell identity.

Atherosclerotic vascular disease is a leading cause of morbidity and mortality in the developed world (7). A critical early step in the development of atherosclerosis is endothelial injury and the resulting endothelial dysfunction which stimulates an infiltration of leukocytes into the vessel wall (8). In the later stages of atherosclerosis, plaque endothelial cells (ECs) are subject to an adverse microenvironment characterized by hypoxia and proinflammatory stimuli, greatly affecting their function as endothelial barrier (9). To date, some instances of specific lncRNAs involved in the maintenance of EC functions, particularly angiogenesis, have been uncovered (10). For example, MALAT1, a highly abundant lncRNA, has been implicated in EC proliferation, migration and tube formation (10). Several lncRNAs have been linked to the control of hypoxia responses via the modulation of hypoxia-inducible factor 1α (HIF1α) activity in the context of tumor hypoxia (11), representing molecular mechanisms that could also be active in hypoxic atherosclerotic plaques. However, the diversity, expression dynamics and functions of lncRNAs in endothelial cells remain poorly characterized, in part due to technical limitations of standard RNA-Seq protocols as well as challenges in data analysis, compared to the profiling of protein-coding genes.

In the current study, we characterize the nascent lncRNA profiles of human primary endothelial cells subjected to hypoxia by performing GRO-Seq. We demonstrate that hypoxia has extensive genome-wide effects on the non-coding transcriptome, with hundreds of known and novel lncRNAs being differentially expressed. We further study the correlation of lncRNAs with coding gene expression and identify a subset of highly stable lncRNAs that are differentially regulated in human atherosclerotic lesions.

MATERIALS AND METHODS

Cell Culture

Human umbilical vein endothelial cells (HUVECs) from 3 different donors were isolated from umbilical cords obtained from the maternity ward of the Kuopio University Hospital and used at passages 5–8. The 4th replicate represents a pool of HUVECs purchased from Gibco and used as passage 10 (potential of >16 population doublings guaranteed). The gene expression profiles of all donors correlated strongly. This work was carried out in accordance with the recommendations of the Research Ethics Committee of the Hospital District of Northern Savo, Kuopio, Finland. Informed written consent was received from all participants and the experiments were performed in accordance with the relevant guidelines and regulations.

HUVECs were maintained in endothelial cell growth medium (EGM; basal medium with SingleQuots supplements CC-4133; Lonza) on cell culture flasks coated with 10 g/ml fibronectin (Sigma, St Louis, MO, USA) and 0.05% gelatin and maintained at 37°C and 5% CO_2. Hypoxia was induced in Ruskinn Invivo2 400 hypoxia workstation (Baker Ruskinn) in the presence of 1% O_2 and 5% CO_2 for 8 h. The 8 h timepoint was chosen to provide representation of early and late responses (12). Moreover, short exposure to hypoxia tends to promote cell survival and growth, while prolonged exposure to hypoxia leads to cell death (13). For adenoviral overexpression, cells were treated with AdCMV (empty vector), AdHIF1α and AdHIF2α (constitutively active forms of HIFs) (14).

GRO-Seq and RNA-Seq

For GRO-Seq nuclei isolation, cells were treated with cycloheximide (0.1 mg/ml) for 10 min, PBS-washed and incubated in 10 ml of swelling buffer [10 mM Tris-HCl, 2 mM $MgCl_2$, 3 mM $CaCl_2$, and 2 U/ml SUPERase Inhibitor (Thermofisher, Waltham, MA, U.S.A.)] for 5 min on ice. Cells were scraped and pelleted for 10 min at 400 × g and resuspended in 500 µl of swelling buffer supplemented with 10% glycerol. Subsequently, 500 µl of swelling buffer supplemented with 10% glycerol and 1% Igepal was added drop by drop to the cells under gentle vortexing. Nuclei were washed twice with lysis buffer (10 ml of swelling buffer supplemented with 0.5% Igepal and 10% glycerol), and once with 1 ml of freezing buffer (50 mM Tris-HCl pH 8.3, 40% glycerol, 5 mM $MgCl_2$ and 0.1 mM EDTA). Nuclei were counted manually using a Bürker chamber after Trypan Blue staining, centrifuged at 900 × g for 6 min and suspended to a concentration of 5 million nuclei per 100 µl of freezing buffer, snap-frozen in liquid nitrogen and stored −80°C until run-on reactions. For the run-on reaction, the nuclear run-on reaction buffer [NRO-RB; 496 µM KCl, 16.5 µM Tris-HCl, 8.25 µM MgCl2 and 1.65was preheated to 30°C. Then each ml of the NRO-RB was supplemented with 1.5 mM DTT, 750 µM ATP, 750 µM GTP, 4.5 µM CTP, 750 µM Br-UTP (Santa Cruz Biotechnology, Inc., Dallas, Texas, U.S.A.) and 33 µl of SUPERase Inhibitor (Thermofisher Scientific, Waltham, MA, U.S.A). Fifty microliters of the supplemented NRO-RB was added to 100 µl of nuclei samples, thoroughly mixed and incubated for 5 min at 30°C. RNA was then harvested by phenol-chloroform extraction [TRIzol LS (Thermofisher Scientific, Waltham, MA, U.S.A)].

For total RNA isolation, cells were treated with cycloheximide (0.1 mg/ml) for 10 min, PBS-washed and scraped into lysis buffer [1x Mammalian Polysome Buffer (Epicenter, Madison, Wisconsin), 1% Triton X-100, 1 mM DTT, 250 U/ml SUPERase Inhibitor, 7.1 U/ml Turbo DNase (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.) and 0.1 mg/ml Cycloheximide] on ice. To assure complete lysis, the lysates were drawn up and expelled 4 times through a sterile 22–25 gauge needle. The cleared lysate was then treated with 10% SDS, snap-frozen in liquid nitrogen and stored −80°C.

Abbreviations: EC, endothelial cell; GRO-Seq, global run-on sequencing; HIF, hypoxia-inducible factor; HUVEC, human umbilical vein endothelial cell; lncRNA, long non-coding RNA; p-lncRNA, promoter-associated lncRNA; eRNA, enhancer-associated lncRNA; SE, super-enhancer.

GRO-seq libraries were subsequently prepared as previously described (15). The run-on products were treated with DNAse I according to the manufacturer's instructions (TURBO DNA-free Kit, Thermofisher, Carlsbad, CA, U.S.A.), base hydrolysed (RNA fragmentation reagent, Thermofisher, Carlsbad, CA, U.S.A.), end-repaired, and immuno-purified using anti-Br-UTP agarose beads (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA).

Total RNA with size >200 nt for sequencing libraries was purified using the Zymo RNA Clean and Conc kit (Zymo Research, Irvine, California, U.S.A.) and rRNAs were removed using the Ribo-Zero Gold rRNA Removal Kit (Illumina, San Diego, CA, U.S.A.). This was followed by fragmentation (RNA fragmentation reagent, Thermofisher) and dephosphorylation.

Subsequently, both RNA-seq and GRO-seq RNA fragments were poly-A tailed (PolyA polymerase, New England Biolabs, Ipswich, MA, USA) according to manufacturer's instructions, followed by circularization and re-linearization. The cDNA templates were PCR amplified (Illumina barcoding) for 11–16 cycles. The GRO-seq libraries were size selected to be 180–300 bp in length. The RNA-seq libraries were selected to be 190–350 bp in length. The final, strand-specific libraries were quantified (Qubit dsDNA HS Assay Kit on a Qubit fluorometer, Thermofisher, Carlsbad, CA, USA) and pooled for 50 bp single-end sequencing with Illumina Hi-Seq2000 (GeneCore, EMBL Heidelberg, Germany).

Mapping and Data Processing

Sequencing results were trimmed to remove 3' A-stretches originating from the library preparation and poor quality reads were filtered out (minimum 97% of bp over quality cutoff 10). Reads were aligned to the hg19 genome using bowtie allowing up to two mismatches and reporting only one alignment for each read. RNA-Seq data was mapped using STAR v2.5.4b to GRCh37/hg19 reference genome (16) with ENCODE standard options for long RNA-seq pipeline (1). Each sequencing experiment was visualized using custom tracks for the UCSC Genome Browser. R version 3.4.0 was used to filter the data, make calculations and create plots.

Detection of Long Non-coding RNA

De novo lncRNA detection from GRO-Seq was performed using using Homer V4.9 (17) "findPeaks.pl" - algorithm with "–groseq" option. To separate lncRNAs from overlapping Refseq genes, "mergePeaks"-command was used with "-strand" option. Refseq annotated ncRNAs were further separated from protein coding genes based on "NR_" accession prefix (and further exclusion of those with "protein coding" annotation). To further divide lncRNAs to known and novel lncRNAs, the coordinates were intersected with the non-codeV5 database (18). When the overlap with database was over 70%, the lncRNA was assigned the non-coding RNA ID. SnoRNAs, ScaRNAs, and mature miRNAs were removed from the data.

Data Analysis

The differential gene expression analysis was performed for transcripts that were expressed (RPKM >0.5) in at least 3 samples among the 8 studied ($n = 4$ for normoxia and hypoxia) using EdgeR (19). Similarly, the correlation between the lncRNA expression and the closest coding gene was performed for the coding genes that were expressed (RPKM >0.5) in at least 3 samples among the 8 studied. This criteria has been defined in order to remove low counts in the libraries to improve the sensitivity and the precision of the differential genes expression (20). Moreover, this threshold was selected because, for GRO-Seq, reads are counted throughout the gene body, which represents more total reads per genes than RNA-seq (12, 15, 21, 22). Differentially expressed genes were defined as transcripts that exhibited over 2-fold change in expression compared to control and FDR < 5%. Ingenuity Pathway Analysis (IPA; QIAGEN) or DAVID 6.8 (23, 24) was used to analyse the pathways and gene ontologies enriched among the differentially regulated genes under hypoxia (25).

LncRNAs were divided to promoter- and enhancer associated transcripts by measuring the average signal of H3K4me3 and H3K4me1 calculated from the datasets GSE29611 and GSE39089 around 1 kb of transcriptional start site. A $\log_2$-ratio of H3K4me3 to H3K4me1 was calculated and positive ratios were assigned as promoter-associated transcripts and negative ratio as enhancer-associated (enhancer RNA) transcripts. Normal enhancers and super-enhancers (SEs) were detected from the normoxia and hypoxia datasets of public H3K27ac ChiP-Seq data (26) using the homer algorithm "findPeaks.pl" with "-style histone" and "-style super" settings, respectively. The eRNA expression was quantified for the combination of normal enhancers and super-enhancers from GRO-Seq and differential expression was determined using edgeR. SEs exhibiting FDR < 0.05 were selected for further analysis (**Table S1**). The SEs were detected for hypoxia and normoxia and those not overlapping were defined as gained/lost SEs upon hypoxia. To identify the stable lncRNA, we correlated the GRO-Seq and RNA-Seq data from two matching HUVEC donors. Highly stable lncRNAs were defined as a transcript that exhibited a RPKM >2 in RNA-seq. This analysis was performed separately for the two conditions. The expression of stable lncRNAs was compared to previously published microarray data (27) for the lncRNA genes that were represented by the Affymetrix HGU133 Plus2 array.

Human Sample Data

Atherosclerotic segments of femoral arteries from 4 patients with primary atherosclerotic lesions ($n = 4$), 5 patients with restenotic lesions ($n = 5$) were compared with non-atherosclerotic arteries from 4 patients ($n = 4$). The samples were age matched and average age (+SD) of the patients for atherosclerotic plaques and normal mammary artery controls were 70.9(+7.3) and 67.2(+9.1) years, respectively (27). Atherosclerotic samples were collected at vascular atherectomy operations (28). The normal samples represent trimmed ends of mammary arteries isolated during cardiac bypass surgery. Total RNA was isolated, amplified, labeled and hybridized to Affymetrix HGU133 Plus2 microarrays comprising 54 675 probe sets) essentially as recommended by the manufacturer (Affymetrix, Santa Clara, CA, USA) exploiting 3′ IVT Express Kit (Affymetrix, Santa Clara, California, US) for probe preparation and GeneChip® Hybridization, Wash, and Stain Kit (Affymetrix, Santa Clara, California, US) for

hybridization and detection. The analysis of gene expression followed standard procedures including Robust Multichip Average (RMA) data normalization. After RMA normalization (29) of the microarray raw data, a filtering step was applied to remove the weakest signals (intensities lower than 2 × above global background) and from initial 54,675 probe sets 17,589 were included in Significance Analysis of Microarrays (SAM); (30) to identify differentially expressed genes at False Discovery Rate <0.1. The studies were approved by Local Ethical Committee of Kuopio University Hospital under identification number 53/2011 and the subjects gave informed consent.

Data Access

The GRO-Seq data is available under NCBI Gene Expression Omnibus under accession number GSE103945. The public ChIP-Seq datasets analyzed can be found under accession numbers GSE29611 (H3K4me1: GSM733690 and H3K4me3: GSM733673 and H3K27ac: GSM733696) and GSE39089 (H3K4me1: GSM955981 and H3K4me3: GSM955983). The RNA-Seq from AdHIF1α and AdHIF2α overexpression are available under accession GSE98060. The microarray data from human femoral atherosclerotic lesions collected during atherectomy can be found under accession GSE53274 and GSE7307. The RNA-Seq data of hypoxia treated HUVECs and GRO-Seq data of adenovirally overexpressed constitutively active HIF1α and HIF2α have been submitted to under accession number GSE118530.

RESULTS

Hypoxia Regulates the Expression of Hundreds of Coding and Non-coding RNAs on Transcriptional Level

To study the nascent transcriptomes of ncRNAs in primary human endothelial cells, we performed GRO-Seq on HUVECs subjected to 8 h of hypoxia (1% oxygen). Altogether, we identified 33,508 transcripts above the expression threshold of 0.5 RPKM in at least 3 samples. Among these, 468 protein coding transcripts were found upregulated and 57 downregulated at least 2-fold (with false discovery rate below 5%) in response to hypoxia (**Table S2A**). We further analyzed these genes for gene ontology and upstream regulators using Ingenuity Pathway Analysis. As expected, HIF1α signaling and glucose metabolism were among the top canonical pathways enriched (**Table S3)** and HIF1α was identified as the top upstream regulator of the differentially expressed genes (**Figure 1A**). This confirms that a significant fraction of hypoxia-regulated genes are regulated on a transcriptional level.

In addition to protein-coding genes, we identified 1,763 differentially regulated lncRNAs in our analysis (applying a false discovery rate threshold of 5%), with 544 upregulated and 350 downregulated more than 2-fold. (**Table S2B**, **Figure 1B**). Eight hundred and eight of these lncRNAs corresponded to known lncRNAs (RefSeq or NONCODE) (**Figure 1C**) and 955 were novel, previously uncharacterized lncRNAs. Altogether, this demonstrates that hypoxia regulates a markedly larger set of non-coding RNAs than protein coding transcripts.

Promoter-Associated lncRNAs Are Enriched for Upregulated Transcripts

To further classify the lncRNAs based on their epigenomic features, we divided the transcripts to promoter- and enhancer-associated lncRNAs according to higher enrichment for H3K4me3 and H3K4me1 histone marks, respectively (31). Altogether, we were able to define 707 promoter-associated lncRNAs (p-lncRNA) and 1056 enhancer RNAs (eRNAs) (**Figure 2A**). Surprisingly, majority (90%) of the differentially regulated p-lncRNAs were induced upon hypoxia stimulus, in contrast to eRNAs that showed an approximately equal distribution of expression change directions (**Figure 2A**). To identify possible mechanisms underlying the differences in expression profiles of different types of lncRNAs, we performed *de novo* motif analysis near their transcription start sites. Clear differences were seen among the top transcription factor motifs enriched, with JUN/AP-1, MAF and NKX3 transcription factor motifs being enriched at the start sites of eRNAs, whereas NRF1, E2F, and SP1 were enriched at the promoters of p-lncRNAs (**Figure 2B**).

The transcriptional response to hypoxia is known to be controlled by the two master regulators, HIF1α and HIF2α, which are able to collaborate with distinct transcription factors (14, 32). To see whether differences in the usage of different HIF α-subunits contributes to differential activation of lncRNA subtypes, we further assessed the regulation of p-lncRNAs and eRNAs by overexpressing the constitutively active HIF1α and HIF2α proteins in HUVECs for 48 h. Western blot analysis of the HUVEC lysates demonstrated high expression of HIF1α and HIF2α protein levels relative to the AdCMV-transduced control cells (**Figure S1**). The GRO-Seq analysis demonstrated that HIF2α overexpression led to more induced p-lncRNAs (54%−140/260) compared to HIF1α (40%−104/260) (**Figure 2C**). The higher induction of lncRNA expression by HIF2α was further confirmed using RNA-Seq (**Figure 2D**).

Hypoxia Response Leads to Significant Regulation of Super-Enhancer Activity

Recent studies have suggested that the expression of cell type-specific genes is controlled through clusters of enhancers called super-enhancers (33). There is also accumulating evidence that these enhancer-dense genomic regions play key roles in cellular response to stimulus (34, 35). To study how hypoxia affects SEs, we used H3K27ac profiles from HUVECs subjected to normoxia and hypoxia for 24 h. Altogether, 1058 and 799 SEs were identified under normoxia and hypoxia conditions, respectively, which were highly concordant with the number of published basal HUVEC SEs (33). As the ChIP-Seq data lacked sufficient replicates and GRO-Seq data can also serve as a reliable indicator of enhancer activity, we used our GRO-Seq data from 4 independent replicates to identify which of these SEs are hypoxia-regulated. The results show that hypoxia led to significant regulation of 573 SEs (**Table S1**, **Figure 3A)** by inducing the expression of 358 SEs and repressing the activity of 215. Interestingly, the induced SEs were found nearby differentially expressed coding genes related angiogenesis (e.g., PGF, MMP2, DLL4, EGFL7, and TGFB1), regulation of apoptosis

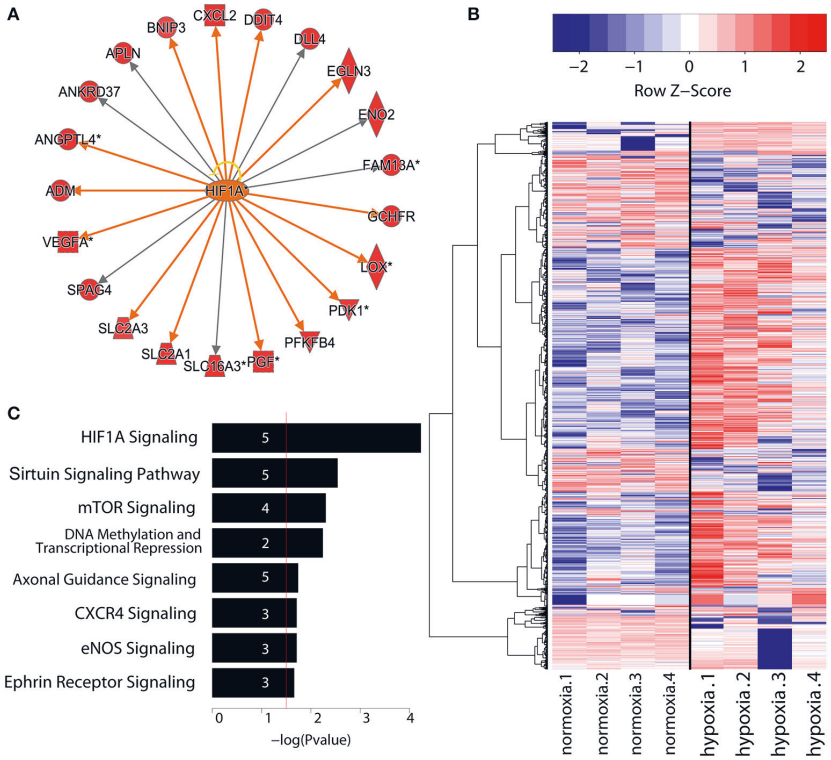

FIGURE 1 | (A) Identification of top upstream regulators responsible for gene expression changes during EC differentiation confirmed the central role of HIF1α.Threshold was defined as FDR < 0.05 and $\log_2$(FC) > 1.5. Activation of downstream genes by HIF1α is indicated with orange arrows, whereas gray arrows signify unknown direction of effect. Yellow arrow indicates inconsistent findings with the state of downstream molecule. Diamond shape represents enzyme, square shape: cytokine, dotted square shape: growth factor, triangle shape: kinase, trapezoid shape: transporter, and circle shape: other. Network graph obtained using IPA (QIAGEN). **(B)** Heatmap showing the expression values in $\log_2$(RPKM) of differentially regulated lncRNAs (FDR < 5%). Throughout the paper, the normalization is in RPKM. Rows are hierarchically clustered using Ward's least absolute error with Minkowski distance. And colors are row scaled. **(C)** Gene ontology analysis of lncRNAs under hypoxia condition generated with IPA (QIAGEN). Numbers in the bars indicate the amount of genes included in the pathways.

(e.g., NOTCH1, SOX4, ANKRD1, and DUSP6), cell adhesion (e.g., CD34, CDH5, VWF, and CTNNB1), and transcriptional activation (e.g., MEF2A, SOX4, EGLN1, MAFK, ASH2L, and TCEA2), whereas the downregulated SEs were close to regulators of peptidyl-tyrosine dephosphorylation (e.g., DUSP6, PTP4A2, PTPN14, and PTPN1) and genes involved in signal transduction (e.g., IL15, IL15RA, and MAP2K6) and GTPase activity (e.g., ARHGAP7/18/24/29; **Table S4**, **Figures 3B,C**). Notably, for 20% of SEs the closest RefSeq gene was a lncRNA, as exemplified by MALAT1 (**Figure 3D**) and LUCAT1.

LncRNAs Expression Is Co-regulated With Nearby Protein-Coding Genes and Mostly Represented by Unstable RNAs

We next correlated the fold change of lncRNAs and their nearby coding genes in response to hypoxia. The results demonstrated a good correlation with the coding gene that was stronger for eRNAs than for p-lncRNAs (**Figure 4A**). This supports the concept that coding genes could be co-regulated through sharing of regulatory motifs or cis-regulation mediated by lncRNAs (36).

To infer functions of the identified lncRNAs, we applied the guilt-by-association principle: if lncRNA shows an expression profile that correlates with nearby protein-coding genes involved in a given function, the lncRNA may be involved in the same function. Gene ontology analysis indicates that genes expressed in close proximity to hypoxia-regulated lncRNAs are mostly involved in angiogenesis and cell migration, as exemplified by the induction of pathways such as ephrin signaling, HIF1α and the VEGF family ligand-receptor interactions pathway (**Figure 4B**). This analysis thus predicts that lncRNAs could be important in hypoxia-mediated changes in endothelial cell signaling and function.

LncRNAs could also mediate *trans*-effects, which likely require the lncRNA transcript to be abundant or long-lived (37–39). To identify this subgroup of stable lncRNAs, we compared the GRO-Seq expression levels to RNA-Seq data from matching

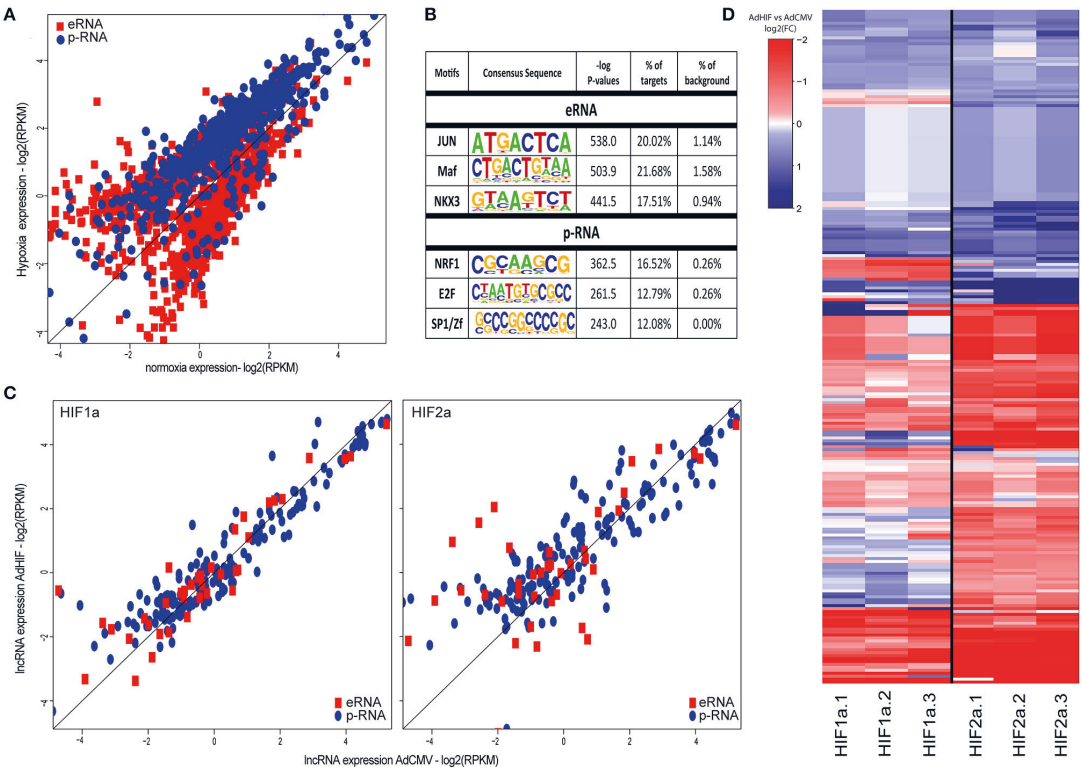

FIGURE 2 | (A) Scatter plot of the expression values (log_2 RPKM) of differentially expressed lncRNAs in response to hypoxia (FDR < 5%). Red squares represent lncRNAs exhibiting high H3K4me1 signal (eRNAs), blue dots represent lncRNAs exhibiting high H3K4me3 signal (p-RNAs). **(B)** DNA-binding motif enrichment analysis at the transcription start sites of the two lncRNA categories (±500 bp). All differentially regulated lncRNAs were used as background. **(C)** Scatter plot showing the nascent lncRNA expression (log_2 RPKM of GRO-Seq signal) after adenoviral overexpression of constitutively active forms of HIF1α or HIF2α compared to adenovirus without transgene (AdCMV). Red squares represent eRNAs, blue dots represent p-RNAs. **(D)** Heatmap showing the log_2(FC) of the lncRNAs after adenoviral overexpression of HIF1α or HIF2α compared to adenoviral overexpression of CMV based on RNA-Seq. Rows are hierarchically clustered using Ward's least absolute error with Euclidian distance.

HUVEC donors. Here, RNA-Seq is expected to reflect the stable lncRNA pool within cell, whereas GRO-Seq is able to display all nascent transcripts irrespective of their half-life. Altogether, 13% of the lncRNAs seen in GRO-Seq were detected using RNA-Seq, and majority of these were associated with promoter-signature (among the 236 detected lncRNAs, 180 were promoter-associated lncRNAs and 56 were enhancer-associated lncRNAs). This was in contrast to protein-coding genes, for which 84% of GRO-Seq-detected genes exhibited detectable expression in RNA-Seq as well (data not shown). The low fraction of lncRNAs detected using RNA-Seq highlights the advantage of GRO-Seq in capturing a large amount of unstable lncRNAs that would otherwise be missed. Interestingly, the hypoxia regulation of eRNAs (fold change in GRO-Seq) exhibited a higher correlation with RNA-Seq than that of p-lncRNAs, suggesting that larger fraction of stable p-lncRNAs could be further regulated at the post-transcriptional level (**Figure 4C**, **Figure S2**). Supporting this, similarly to mRNAs, the p-lncRNAs have been shown to be targeted by cytoplasmic miRNAs and even act as competing endogenous RNAs (40), whereas the eRNAs are less likely to leave the nucleus.

Regulation of lncRNAs in Human Atherosclerotic Lesions

We subsequently investigated whether the expression of the stable lncRNAs is altered during atherosclerosis. To this end, the atherosclerotic segments of femoral arteries from 4 patients with primary atherosclerotic lesions and 5 patients with restenotic lesions were compared with non-atherosclerotic mammary arteries from 4 patients (27). Altogether 30 hypoxia-regulated lncRNAs from our HUVEC analysis were represented by the probe sets of the microarray and found to be differentially regulated in the atherosclerotic samples compared to the control tissue. This included for example the well-known lncRNA MALAT1 (**Figure 5**) and the lncRNA HYMAI, which has been

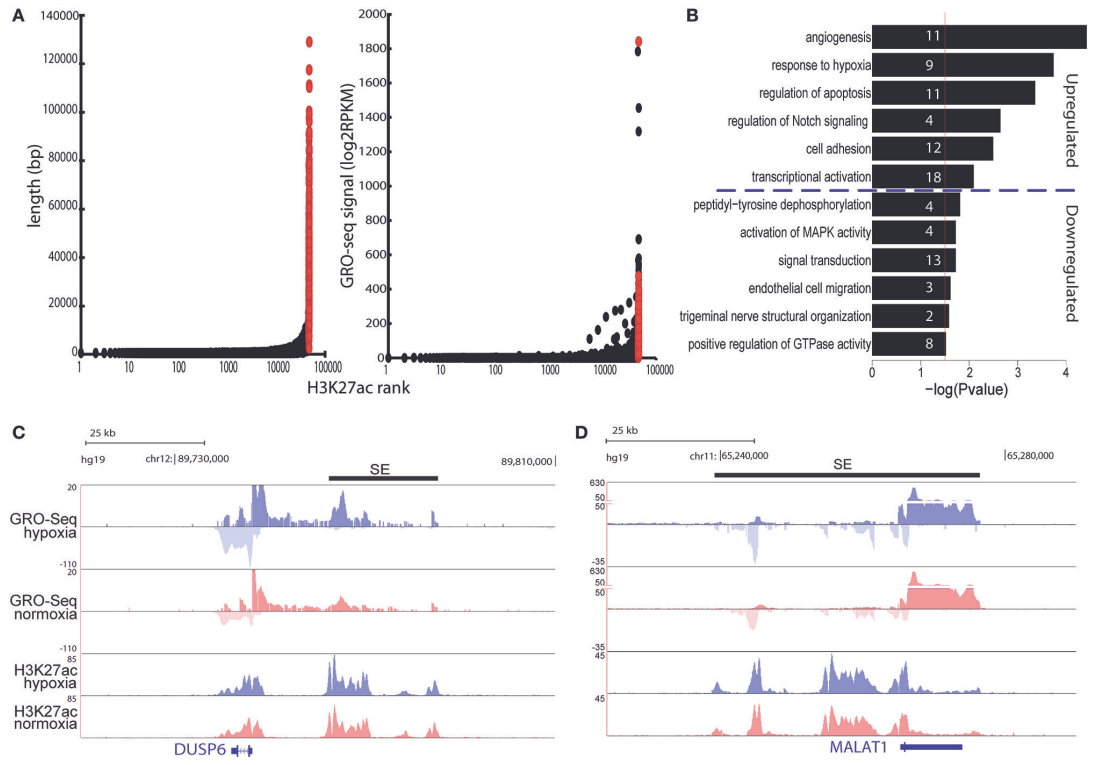

FIGURE 3 | (A) Left: total length of super-enhancers (red) and normal enhancers (black) ranked by increasing H3K27ac signal under hypoxia. Right: total GRO-Seq signal (count per million) for super-enhancers and normal enhancers using the H3K27ac based ranking. **(B)** Gene ontology analysis of the differentially expressed genes located <100 kb from induced and repressed superenhancers. Gene ontology was performed with DAVID. Numbers in the bars indicate the amount of genes included in the pathways. **(C,D)** UCSC genome browser shot images of DUSP6 **(C)** and MALAT1 **(D)** under hypoxia (blue) and normoxia (red). Normalized tag counts are shown for GRO-Seq and ChIP-Seq. Black bars represent the super-enhancer position.

studied in relation to diabetes (41, 42) but not previously reported in association with atherosclerosis or hypoxia. Several lncRNAs with the notable ability to distinguish between healthy and diseased vascular tissue, such as LOC730101, KIAA1656, and LOC339803, remain poorly studied. However, LOC730101 has been implicated in the activation of Wnt/β-catenin signaling (43), a pathway that is critical for endothelial cell proliferation and migration (44). Notably, restenosis samples following the intravascular surgery of the primary lesion formed a separate cluster from the primary lesions, demonstrating further altered lncRNA profiles during intimal hyperplasia and arterial remodeling in the treated arteries. Altogether, our results indicate significant regulation of lncRNA expression in human atherosclerotic lesions which could contribute to atherosclerotic lesion progression or serve as indicators of lesion hypoxia.

DISCUSSION

Currently, over 200,000 unique lncRNAs have been discovered across 50 human tissues or cell lines (45, 46). Here, we expand that list of lncRNAs and contribute to the characterization of hypoxia-regulated lncRNAs in human primary endothelial cells. Tissue hypoxia occurs as a part of diseases such as coronary artery disease and cerebrovascular disease, and cells have evolved complex response programs to try to manage and resolve oxygen deficiency (9). Being the innermost layer of cells in the blood vessel, endothelial cells are directly exposed to alterations in blood composition, such as hypoxia, and play a key role in maintaining vascular homeostasis. In particular, the vasculature responds to hypoxic stress by activating angiogenesis and endothelial cell proliferation and migration (9).

By carrying out GRO-Seq, an RNA profiling technique that targets ongoing transcription and is indifferent to transcript polyadenylation, we were able to detect the differential expression of nearly 1800 lncRNAs in endothelial cells in response to hypoxia. Notably, the number of regulated lncRNAs outnumbered that of differentially expressed protein-coding genes under the same conditions (around 500). The most highly upregulated lncRNAs included many that were novel, as well as several lncRNAs described to carry out crucial functions in other types of disease or stress conditions. For example, hypoxia resulted in the marked upregulation of LUCAT1, an anti-apoptotic lncRNA

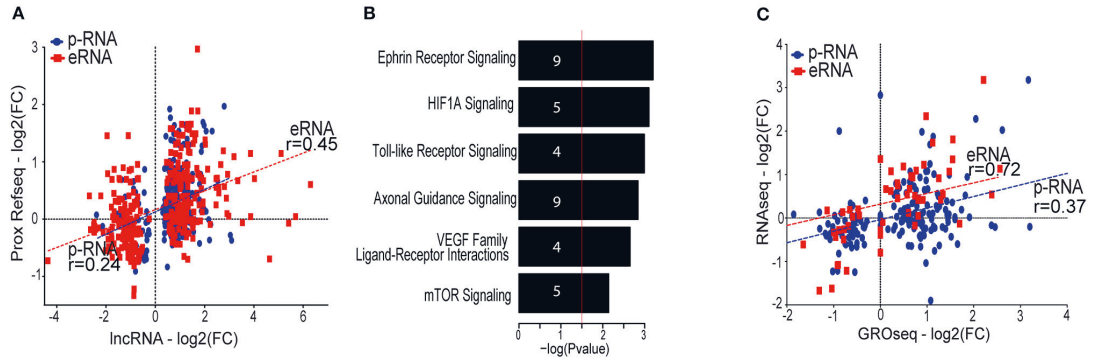

FIGURE 4 | (A) Scatter plot showing the correlation between the lncRNA and the proximal (<100 kb) protein coding genes. Only proximal coding genes exhibiting differential gene expression (FDR value below 5% and a fold change threshold of ±1.5) are displayed. Correlation calculated using Spearman correlation. **(B)** Gene ontology analysis of the differentially expressed proximal coding genes generated with IPA (QIAGEN). Numbers in the bars indicate the amount of genes included in the pathways. **(C)** Scatter plot showing the fold change (log_2) of RNA-Seq compared to the fold change (log_2) of GRO-Seq of the differentially expressed lncRNAs. Transcripts exhibiting low expression (RPKM < 0.5 in <2 samples) were removed from the analysis. Correlation calculated using Spearman correlation.

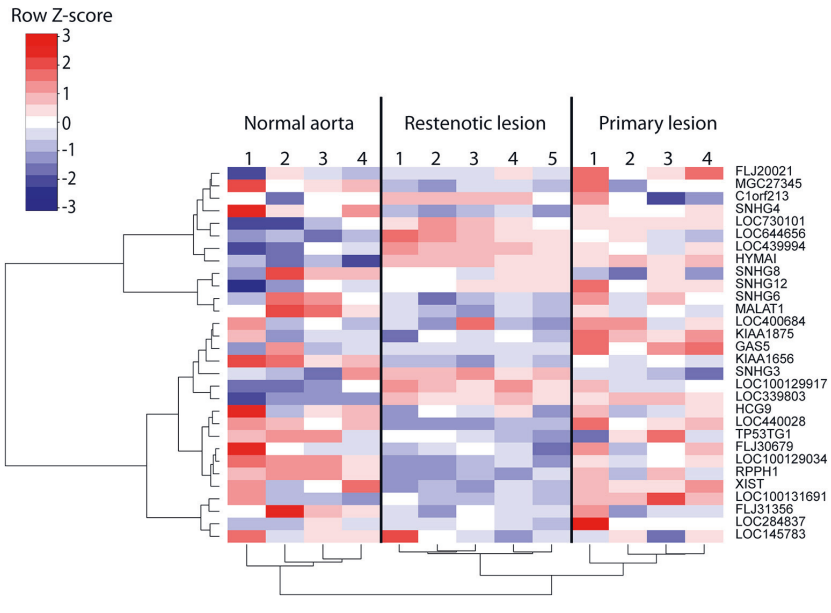

FIGURE 5 | Heatmap of the differentially regulated lncRNAs (FDR < 5%) in 9 samples of human atherosclerotic lesions compared to 4 control regions from non-affected mammary arteries (27). Rows and columns clustered using Ward's least absolute error with Manhattan distance.

recently found to affect DNA methylation by regulating DNMT1 (47), and RASSF1-AS1, which is known to downregulate the level of pro-apoptotic regulator/tumor suppressor RASSF1 (48). Induction was also seen for IDH1-AS1, a lncRNA involved in the control of energy metabolism (49). The induction of these lncRNAs in endothelial cells could promote cell survival and proliferation to facilitate vascular reorganization upon hypoxia.

By integrating GRO-Seq and ChIP-Seq data, we found that a large fraction of H3K4me1-supported lncRNAs (60%) originate from enhancers. This result is in good agreement with the recent FANTOM5 data obtained using cap analysis of gene expression

(CAGE), which demonstrated that 68% of lncRNAs originate from enhancers rather than from promoters (3). We acknowledge that the classification of lncRNAs based in epigenetic marks is far from perfect with emerging evidence of highly active enhancers displaying H3K4me3-promoter mark (50) and promoters serving as enhancers (51, 52). However, this classification allowed us to distinguish that promoter associated lncRNAs and mRNAs are more prone to gene activation in response to hypoxia, compared to enhancer-associated lncRNAs that displayed equal level of transcriptional activation and repression. Our results demonstrate that promoter activation is more evident upon HIF2α activation but also likely involves the differential usage of collaborating transcription factors. To this end, the most highly enriched transcription factor motifs for promoter- and enhancer-associated lncRNAs were clearly distinct. Regions near eRNAs exhibited strongest enrichments for AP-1/Jun and NKX3, factors previously implicated in the control of vascularization (53, 54), and MAFA, a known regulator of energy homeostasis that can act both as activator and repressor of transcription (55). In line with their genomic origin, p-lncRNAs revealed enrichment for motifs known to be promoter-proximal, such as binding motifs for Sp1 and NRF1 (56, 57). Moreover, the most highly overrepresented motifs found near p-lncRNAs (NRF1, E2F, and Sp1) all bind factors associated with cell growth and proliferation (58–60), in line with observations that short-term and/or moderate hypoxia stimulates endothelial cell proliferation (13). We have also recently shown that HIF2α contributes more to the regulation of proliferation-related genes in endothelial cells compared to HIF1α (14). Altogether, our data suggests that HIF2α might collaborate with other transcription factors to mediate preferential activation of p-lncRNAs. Future studies are needed to disentangle the complex mechanisms of transcriptional regulation and the collaborative networks of transcription factors in response to hypoxia.

Our data shows that hypoxia leads to a remodeling of the super-enhancer landscape, with induction and repression of SE activity in the vicinity of genes known to be critical for endothelial cell function. To this end, the SE activity was significantly regulated next to several genes encoding for angiogenic factors, such as EGFL7 (61), DLL4 (62), MMP2 (63), and TGFB1 (62). In accordance with this, SE activation was also seen near several transcriptional regulators, for example MEF2, which is known be a transcriptional effector of VEGF responsible for activating DLL4 expression to drive sprouting angiogenesis (64). On the other hand, decreased SE activity was detected near several genes encoding for Rho GTPase-activating proteins, notably including the known angiogenesis inhibitors ARHGAP7 (DLC1) (65) and ARHGAP18 (66), and near several protein tyrosine phosphatase genes, including PTPN14 and PTPN1 (PTP1B), which have been reported to negatively modulate angiogenesis by regulating VEGF receptor signaling (67, 68). Similarly to protein coding genes, the regulation of SE activity was also evident next to functionally notable lncRNAs, such as the angiogenic lncRNA MALAT1 (10, 69). Thus, profiling enhancer activity by GRO-Seq yields valuable candidate regions for genetic elements that mediate endothelial cell functional responses.

Taken together, our data indicates that dynamic changes in the SEs likely serve an important role in orchestrating the growth of new blood vessels in response to hypoxia. Importantly, the modification of endothelial cell responses through the alteration of SE activity also represents a possible therapeutic approach. For example, promising results have been obtained in modulating endothelial cell inflammatory responses by targeting SEs through the inhibition of bromodomain and extra-terminal domain (BET) factors, a family of transcriptional co-activators and elongation factors (34). Crucially, SEs seem to be more sensitive to BET perturbation than typical enhancers, and the expression of their nearby genes seems to be preferentially affected (70), making BET perturbation a good therapeutic approach.

To evaluate the expression of hypoxia-responsive lncRNAs *in vivo*, we explored the correlation between lncRNA regulation in endothelial cells and human advanced atherosclerotic lesions. Other studies have previously reported on the importance of the lncRNA expression in cardiovascular development and pathophysiology (71–73), and it has been suggested that lncRNAs could serve as circulating biomarkers of endothelial injury associated with vascular diseases (10, 74). Considering the differential expression of the lncRNAs alongside the expression of coding genes and other markers could provide improved insight into the disease state of the patients, for example by helping pinpoint the activation of different types of cellular stress responses. Our results revealed several lncRNAs, such as HYMAI, LOC730101, KIAA1656, and LOC339803 that are transcriptionally induced in endothelial cells during hypoxia and overexpressed in human atherosclerotic plaques compared to control artery samples. Little is currently known about these lncRNAs; however, LOC730101 has previously been implicated in cell survival during bioenergetic stress (75). Moreover, we show that MALAT1 is downregulated in restenotic vasculature and atherosclerotic lesions. MALAT1 overexpression was first described in cancer (76) and in response to 24 h hypoxic stimulus (77, 78). However, there is increasing evidence that the direction of MALAT1 deregulation is dependent on the disease context (79) and recently MALAT1 was described to be downregulated in tissue samples from atherosclerotic coronary artery plaques (80). This is in line with our findings, were MALAT1 was downregulated in both primary and restenotic lesions. This also suggests that other factors than hypoxia could contribute to altered expression of MALAT1 in tissues and the regulation of MALAT1 is highly tissue and cell-type specific. Future studies testing the applicability of these lncRNAs for early detection of disease from blood, as well as detailed investigations into their expression profiles and regulatory mechanisms, are envisioned.

In conclusion, we report a nascent transcriptome atlas of human hypoxia-sensitive lncRNAs in primary endothelial cells and identify several lncRNAs with potential for usage as indicators of endothelial hypoxia in human atherosclerotic lesions or other vascular diseases. Future studies are expected to provide more detailed knowledge of the functional roles of the identified lncRNAs in atherogenic processes, thereby potentially helping to establish lncRNAs as future therapeutic or prognostic targets.

AUTHOR CONTRIBUTIONS

PRM, MUK, and SY-H conceived and designed the experiments. PRM, MUK, Tö, NLD, HN, EA, and SY-H performed the data analysis. MB-L, NLD, EA, and HN acquisition of data. PRM, Tö, MUK, and MB-L drafting the manuscript. All the authors interpreted the data and revised the manuscript for content and accuracy.

FUNDING

This research was supported in part by Finnish Academy Centre of Excellence on Cardiovascular and Metabolic Diseases, the Finnish Foundation for Cardiovascular Research (to 160071 to HN, NLD, and MUK), the Finnish Cultural Foundation (grants 00180732 to PRM and 00170757 HN), the Antti and Tyyne Soininen Foundation (to HN), the Ida Montin Foundation (to HN), the Academy of Finland (grants 287478 and 294073 to MUK, and 295094 to MB-L), the Sigrid Jusélius Foundation (to MUK), the Jane and Aatos Erkko Foundation (to MUK), the Diabetes Research Foundation (to MUK), the Fondation Leducq LEAN project (to SY-H) and the ERC Advanced Grant 670951 (to SY-H).

ACKNOWLEDGMENTS

We thank the Sequencing Service GeneCore Sequencing Facility (EMBL, www.genecore.embl.de) for NGS library sequencing and University of Eastern Finland Bioinformatics Center for server infrastructure.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2018.00159/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer UR and handling editor declared their shared affiliation at the time of the review.

III

Profiling nuclear and cytoplasmic non-coding transcriptomes in endothelial cells under hypoxia

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Manuscript

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Atherosclerosis is a major cause of death and impaired living. However, we are far from understanding how vascular cell responses to pro-atherogenic stimuli contribute to the disease process. This thesis investigates how vascular cell transcriptional and post-transcriptional profiles change under stress, revealing unique non-coding RNA expression patterns and their functionality in regulating cellular pathways. These findings pave the way for a better understanding of disease development.

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PUBLICATIONS OF
THE UNIVERSITY OF EASTERN FINLAND
Dissertations in Health Sciences

ISBN 978-952-61-5199-1
ISSN 1798-5706