EGON URGARD

Potential therapeutic approaches
for modulation of inflammatory
response pathways

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LIST OF ORIGINAL PUBLICATIONS

The current thesis includes the following original publications, which will be referred in the text by their Roman numerals.

I. **Urgard, E.**, Reigo, A., Reinmaa, E., Rebane, A., Metspalu, A. 2017 Human basonuclin 2 up-regulates a cascade set of interferon-stimulated genes with anti-cancerous properties in a lung cancer model, *Cancer Cell Int*, 17: 18.

II. **Urgard, E.**, Lorents, A., Klaas, M., Padari, K., Viil, J., Runnel, T., Langel, K., Kingo, K., Tkaczyk, E., Langel, Ü., Maimets, T., Jaks, V., Pooga, M., Rebane, A. 2016 Pre-administration of PepFect6-microRNA-146a nano-complexes inhibits inflammatory responses in keratinocytes and in a mouse model of irritant contact dermatitis, *J Control Release*, 235: 195–204.

III. **Urgard, E.**, Brjalin, A., Langel, Ü., Pooga, M., Rebane, A., Annilo, T. 2017 Comparison of Peptide- and Lipid-Based Delivery of miR-34a-5p Mimic into PPC-1 Cells, *Nucleic Acid Ther* 27(5): 295–302.

Contributions of Egon Urgard to original publications:

Study I: Participated in the study design, performed the experiments, analyzed the data and wrote the manuscript.

Study II: Contributed to the study design, performed most of the cell culture experiments or repeated them for validation, participated in the mouse experiments, the data analysis and interpretation and writing the manuscript.

Study III: Contributed to the study design, performed most of the cell culture experiments or repeated them for validation, participated in the data analysis and interpretation and writing the manuscript.

LIST OF ABBREVIATIONS

A549	Adenocarcinomic human alveolar basal epithelial cells
AC	Adenocarcinoma
AD	Atopic dermatitis
AGO	Argonaute
ASO	Antisense oligonucleotide
ARHGAP1	Rho GTPase Activating Protein 1
AXL	AXL receptor tyrosine kinase
BCL	B-cell lymphoma
BEAS-2B	Human bronchial epithelial cells
BNC1/2	Basonuclin 1/2
CARD10	Caspase recruitment domain family member 10
CCL	C-C Motif Chemokine Ligand
CDC25a	Cell division cycle 25a
CDK	Cyclin dependent kinase
CLDN1	Claudin-1
CPP	Cell-penetrating peptide
CTCL	Cutaneous T cell lymphoma
DGCR8	Drosha-DiGeorge syndrome critical region 8
FDA	Food and Drug Administration
FOSL1	FOS Like 1, AP-1 Transcription Factor Subunit
GalNAc	N-Acetylgalactosamine
HCC	Hepatocellular carcinoma
HEK293	Human embryonic kidney cell line
HCV	Hepatitis C virus
ICAM1	Intercellular Adhesion Molecule 1
ICD	Irritant contact dermatitis
IFITM	INF-induced transmembrane protein
IFN	Interferon
IκB	Inhibitors of κB
IL	Interleukin
IRAK1	IL-1 Receptor Associated Kinase 1
IRF	IFN regulatory factor
ISG	IFN stimulated gene
JAK	Janus kinase
LF2000	Lipofectamine 2000
LPS	Lipopolysaccharide
miRNA	microRNA
MET	Mesenchymal-epithelial transition factor
MYC	v-myc avian myelocytomatosis viral oncogene homolog
LNA	Locked nucleic acid
NA	Nucleic acid
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells

NSCLC	Non-small cell lung cancer
NUP210	Nucleoporin 210
OAS	2,5-oligoadenylate synthetase
pAntp	Peptide from Drosophila melanogaster Antennapedia
PD1	Programmed death 1
PD-L1	Programmed death-ligand 1
PDCD4	Programmed cell death 4
PDGFRA	Platelet derived growth factor receptor alfa
pDNA	plasmid DNA
PF	PepFect
PMA	Phorbol 12-myristate 13-acetate
PNA	Peptide nucleic acid
PPC-1	Primary prostatic carcinoma cell line
PTEN	Phosphatase and tensin homolog
RAS	Rat sarcoma oncogene
RISC	RNA-induced silencing complex
SCC	Squamous cell carcinoma
SH2	Src homology 2
shRNA	small hairpin RNA
siRNA	small interfering RNA
SIRT1	Silent information regulator 1
SOCS1	Suppressor of cytokine signaling 1
STAT	Signal transducers and activators of transcription
Tat	Trans-activator of transcription
TLR	Toll-like receptors
TNF-α	Tumor necrosis factor-α
TP10	Transportan 10
TP53	Tumor protein 53
TRAF	TNF receptor associated factor
TRBP	Transactivation response element RNA-binding protein
TSG	Tumor suppressor gene
XAF1	XIAP associated factor 1
XIAP	X-linked inhibitor of apoptosis protein

INTRODUCTION

Survival potential of multicellular organisms is often determined at the level of subtle changes in their orchestrated defense and regeneration mechanisms. At the frontline for these processes are epithelial tissues being remarkable for their plasticity not only through the evolution from the simplest to the most sophisticated, but also through the lifetime of a single organism. Among other epithelial tissues, those covering the airways and skin together comprise the largest area and are the first line of defense being affected by environmental factors and many pathogens. When failed, several diseased conditions may develop, including acute and chronic inflammation and cancers. Remarkably, in certain cases, the shift towards cancerous changes can be related to chronic inflammation.

The current thesis aims to describe some of the mechanistic aspects involved in the development of cancer and, on the other hand, it is focused on elucidation of the obstacles associated with the development of particular type of novel therapeutic approaches.

First, we investigated functional pathways influenced by Basonuclin 2 (BNC2), a novel zinc finger type transcriptional regulator. Being found downregulated in multiple cancers, BNC2 was chosen for our further studies in respiratory tract derived epithelial cells and we aimed to unfold its role in protection mechanisms against cancerous processes.

Two other research articles included in the thesis are focused on targeting the inflammation- and cancer-related cellular pathways using miRNAs. The therapeutic potential of miRNAs has been proposed earlier, however, the biggest obstacle is still the lack of efficient, safe and specific delivery methods for miRNAs mimics and inhibitors. In addition, there is also a question whether the influence of individual miRNAs can be considered sufficiently strong for therapeutic effect. In our study we used cell penetrating peptides PepFect6 (PF6) and PepFect14 (PF14) and tested their capacity to deliver miRNA mimics in multiple *in vitro* cell culture systems and in local *in vivo* delivery into inflamed skin. Second, our experiments were focused on gathering additional information about anti-inflammatory potential of miR-146a and tumor suppressor properties of miR-34a.

1 REVIEW OF THE LITERATURE

1.1 Inflammatory response pathways

Inflammation is a protective response to injury and infection with the purpose of repairing damage and returning tissue to a healthy state (Dunster 2016). It is a complex process that involves inducers (infection or damage) and is carried on by innate immune cells (mast cells, dendritic cells, macrophages) identifying the affected tissue and inflammatory mediators, such as cytokines and chemokines (Medzhitov 2010). The type of pathway induced under given conditions greatly depends on the nature of the triggers. For example, after recognition of pathogens via the receptors of the innate immune cells, such as Toll-like receptors (TLRs), bacterial infections trigger innate immune response by activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), while viral infections typically induce the production of type I and III interferons (IFNs) (Mogensen 2009, Medzhitov 2010).

The inflammatory response requires the activation of a complex transcriptional program and involves dynamic regulation of hundreds of genes. Among several transcription factors, NF-κB (Hayden and Ghosh 2012) and signal transducers and activators of transcription proteins (STATs) (Stark and Darnell 2012) serve as key modulators of inflammatory response pathways. In addition to the regulation of acute phase inflammation, unbalanced long-term activation of these factors could lead to chronic inflammation, characteristic for instance for allergic diseases, as well as may lead to cancer (Porta et al. 2009).

1.1.1 JAK-STAT pathway

The Janus kinase (JAK)/STAT pathway plays an important role in transferring of signals from cell-membrane receptors to the nucleus (Seif et al. 2017). It is upstream of multiple cellular activities, such as proliferation, differentiation, migration, apoptosis and cell communication or complex biological processes, including inflammation, immune response and cancer (Moresi et al. 2019). Four JAK (JAK1, JAK2, JAK3, tyrosine kinase 2 (TYK2)) and seven STAT (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, STAT6) family members have been identified in mammals (Villarino et al. 2017). Initially, the JAK/STAT pathway was identified as a response to interferon-gamma, however, later, several other extracellular polypeptide signals have been found to activate it. JAK/STAT proteins are ubiquitously expressed and different combinations of them respond to specific cytokines or growth factor signals (Moresi et al. 2019).

The activation of JAK/STAT signaling pathway is initiated by binding of an extracellular ligand, such as a growth factor or cytokine, to its trans-membrane receptor, which activates a receptor-associated JAK tyrosine kinase (Kisseleva et al. 2002). Activated JAK subsequently induces phosphorylation of specific

tyrosine-based motifs in the cytoplasmic tails of the receptors, which provide docking sites for Src homology 2 (SH2)-containing STATs, as well as for other proteins with SH2 domains (Jatiani et al. 2010). Subsequent phosphorylation of STATs leads to their homo- or heterodimerization and translocation to the nucleus, where they bind to the specific DNA sequences of the promoters of target genes, thereby either activating or repressing transcription, often in cooperation with co-activators and co-repressors (Platanias 2005, O'Shea et al. 2013) (Figure 1). The most established interaction of STATs with other transcription factors has been shown for members of the interferon regulatory factor (IRF) family (Tamura et al. 2008) and with NF-κB (van Boxel-Dezaire et al. 2006).
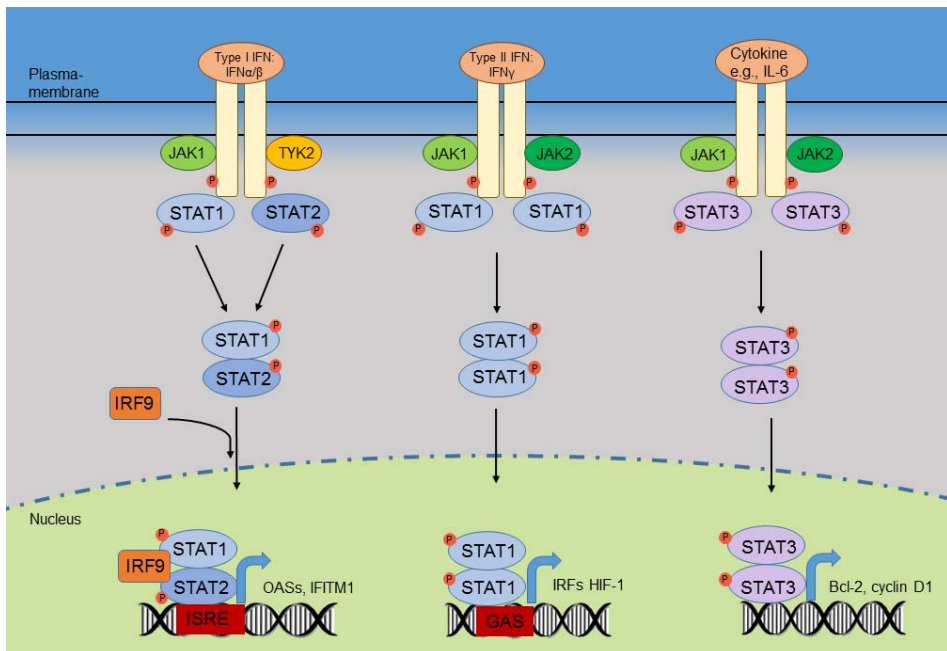
Figure 1. JAK/STAT signaling pathway. The interferon family of ligands activates the receptor-associated protein JAK1 and tyrosine kinase 2 (TYK2), which then phosphorylate the cytoplasmic STAT1/2. Phosphorylated STAT1 and STAT2 form heterodimers, which assemble with IRF9. Formed STAT1-STAT2-IRF9 complex translocates to the nucleus and binds to DNA consensus sequences, known as IFN-stimulated response elements (ISREs), thereby directly activating the transcription of interferon stimulated genes (ISGs). Type III-interferons induce the formation of STAT1–STAT1 homodimers that translocate to the nucleus and bind gamma-interferon activation site (GAS) elements that are present in the promoter of certain ISGs, thereby initiating the transcription of these genes. Beside interferons, other ligands, such as interleukin-6 (IL-6), activate STAT3, which leads to activation of anti-apoptotic and pro-survival genes. Figure is prepared according to the information of Platanias and Turner (Platanias 2005, Turner et al. 2014).

STAT-activated genes include ISGs with antiviral properties, such as IFN induced transmembrane protein 1 (*IFITM1*), myxovirus resistance protein (*MX*) family genes, *IRF*s, innate immune receptors, such as retinoic acid-inducible gene I (*RIG-I*) and melanoma differentiation-associated protein 5 (*MDA5*) and many more (Zhao et al. 2009, Schoggins et al. 2011, Stark and Darnell 2012). In addition to ISGs implicated in antiviral effects, many genes that are associated with the regulation of apoptosis are induced, including *IRF*s, 2,5-oligoadenylate synthetase proteins (*OAS*), X-linked inhibitor of apoptosis (XIAP)-associated factor 1 (*XAF1*), pro-apoptotic members of B-cell lymphoma 2 (*Bcl-2*) family and several caspases (Chawla-Sarkar et al. 2003, Clemens 2003). XAF1 has been recognized as an interacting protein of XIAP. It functions as an antagonist of XIAP by rescuing XIAP-suppressed caspase-3 activity, thus promoting cellular apoptosis (Liston et al. 2001). XAF1 has also been implicated a role as a tumor suppressor, as it is ubiquitously expressed in normal tissues, but is absent or detected at low levels in a wide variety of tumor cells and primary carcinomas (Fong et al. 2000, Chung et al. 2007). XAF1 overexpression induces apoptosis and inhibits tumor growth in multiple types of cancer (Tu et al. 2009, Huang et al. 2010, Zou et al. 2012).

In healthy tissue, signaling via the JAK/STAT pathway is well-controlled, regulated at various levels and can be terminated by a negative feedback process involving specific inhibitors. One class of inhibitors comprises the suppressor of cytokine signaling (SOCS) proteins, which terminate signaling by promoting ubiquitination and subsequent degradation of receptors, JAKs and STATs (Seif et al. 2017). Another class of inhibitory proteins consists of the protein inhibitors of activated STATs (PIAS) proteins, which also promote the degradation of receptors and JAK-STAT pathway components (Liu et al. 2013, Clark et al. 2014).

1.1.2 NF-κB pathway

The NF-κB family of transcription factors has an essential role in the regulation of inflammatory responses and is recognized as a set of crucial players in many steps of cancer initiation and progression (Zheng et al. 2011). NF-κB is ubiquitously expressed in the cytoplasm of all cell types. When activated, NF-κB translocates to the nucleus where it regulates the expression of genes playing important roles in immune and stress responses, and thereby impacts several processes, such as apoptosis, proliferation, differentiation and development (Pahl 1999, Oeckinghaus and Ghosh 2009).

In mammals, the NF-κB transcription factor family consists of five members: RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50) and NF-κB2 (p100/p52). NF-κB1 and NF-κB2 are synthesized as large polypeptides (p105 and p100) that are posttranslationally cleaved to generate the DNA binding subunits p50 and p52, respectively (Caamaño and Hunter 2002). All members of the family associate with each other to form distinct transcriptionally active homo- and heterodimeric complexes, which bind to a variety of related target DNA sequences to modulate gene expression (Gilmore 2006).
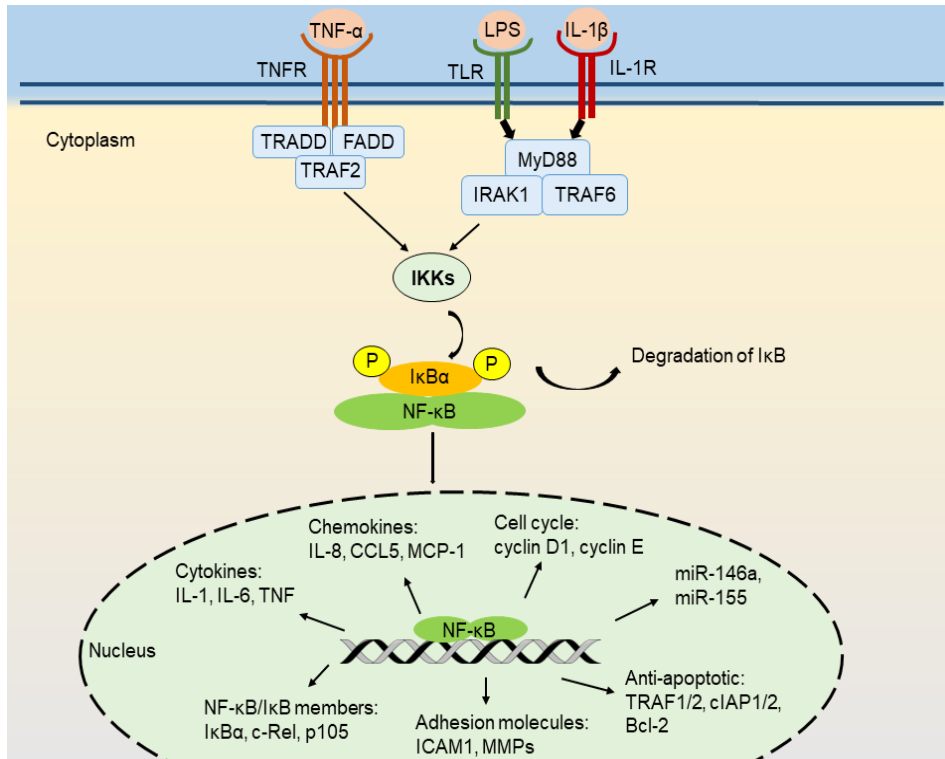
Figure 2. NF-κB signaling pathway. Activation of the NF-κB signaling pathway may occur by lipopolysaccharides (LPS), TNF-α or IL-1β, which bind to Toll-like receptors (TLRs), tumor necrosis factor receptor (TNFR) and IL-1 receptor (IL-1R), respectively. Through recruitment and phosphorylation of a variety of adaptor proteins and kinases, this leads to the activation of IκB kinase complex, which then phosphorylates IκBα. This phosphorylation results in proteasomal degradation of IκBα and translocation of NF-κB homo- and heterodimers to the nucleus and is followed by activation of transcription of a wide variety of target genes. Figure is prepared according to the information of Oeckinghaus and Ghosh, Hoesel and Schmid (Oeckinghaus and Ghosh 2009, Hoesel and Schmid 2013).

Under basal conditions in most cells, NF-κB dimers (e.g. p50-RelA) are kept inactive in cytoplasm by inhibitors of κB (IκB) proteins, which prevent nuclear entry and DNA binding through masking the nuclear localization signal of Rel proteins (Caamaño and Hunter 2002, Espinosa et al. 2015). A large number of external stimuli lead to activation of NF-κB. Typical activating molecules are cytokines, such as tumor necrosis factor α (TNF-α) and IL-1β, and lipo-polysaccharides (LPS), which are bacterial cell wall components. Signaling is mediated through TNF-receptor (TNFR), IL-1 receptor (IL-1R) and Toll-like receptors (TLRs), respectively (Hoesel and Schmid 2013). Stimulation with IL-1β or LPS triggers recruitment of the myeloid differentiation primary response 88 (MyD88), followed by phosphorylation of interleukin 1 receptor associated

kinase 1 (IRAK1) and TNF receptor associated factor 6 (TRAF6). In contrast, stimulation by TNF-α leads to binding of the adaptor TNFR type 1-associated DEATH domain protein (TRADD), which provides an assembly platform for recruitment of the adaptor Fas-associated protein with DEATH domain (FADD) and TRAF2 (Oeckinghaus et al. 2011). Recruitment of these adaptors, in turn, leads to IκB kinase complex activation and degradation of IκB proteins followed by triggering of nuclear translocation of NF-κB and induction of transcription of target genes (Caamaño and Hunter 2002, Oeckinghaus and Ghosh 2009, Liu et al. 2017). The NF-κB induced genes include nearly the entire set of immune guardians: chemokines, cytokines, adhesion molecules, inflammatory mediators and apoptosis inhibitors (Figure 2).

Although NF-κB activity is inducible in most cells, NF-κB can also be detected as a constitutively active nuclear protein in certain cell types, such as mature B cells, macrophages, neurons and vascular smooth muscle cells, as well as a large number of tumor cells (Oeckinghaus and Ghosh 2009).

1.1.3 Inflammation and cancer

Inflammation is a normal and essential process in response to tissue damage resulting from microbial pathogen infection, chemical irritation and/or wounding (Philip et al. 2004). However, when it is unresolved and becomes chronic, the resulting tissue damage can be extensive and disastrous, leading to auto-inflammatory diseases or even cancer (Conway et al. 2016). In many cases, the risk of cancer is increased in chronic infections and inflammatory diseases. For example, gastric cancer and gastric lymphoma are associated with *Helicobacter pylori*, hepatocellular carcinoma is associated with viral hepatitis, colonic cancer with inflammatory bowel disease, and chronic obstructive pulmonary disease (COPD) is a significant risk factor for lung cancer (De Flora and La Maestra 2015, Todoric et al. 2016).

In the process of development of tumors of epithelial origin, the inflammatory component may include diverse leukocyte populations, such as neutrophils, dendritic cells, macrophages, eosinophils and mast cells, as well as lymphocytes (all of which are capable of producing an assorted array of cytokines), chemokines and soluble mediators of cell death, such as TNF-α, IL-1 and IL-6 and IFNs (Todoric et al. 2016).

Tumor Necrosis Factor (TNF) is a major pro-inflammatory cytokine and it is able to act as an endogenous tumor promoter to bridge inflammation and carcinogenesis (Wang and Lin 2008). The primary source of TNF are innate immune cells, such as activated macrophages and neutrophils, but it can also be produced by T and B lymphocytes and natural killer (NK) as well as non-immune cells, including fibroblasts and endothelial cells (Ham et al. 2016). Numerous reports have shown increased TNF expression level in various tumor tissues, such as chronic lymphocytic leukemia, Barrett's adenocarcinoma, prostate cancer,

breast cancer and cervical carcinoma (Ahmed et al. 2001, Ferrajoli et al. 2002, Tselepis et al. 2002, Michalaki et al. 2004, García-Tuñón et al. 2006). TNF is a pleiotropic cytokine exerting both inhibitory and stimulatory effects being either anti- or pro-tumorigenic. Its protective anti-oncogenic effect has been shown in 3'-methylcholanthrene (MCA)-induced skin sarcoma mouse model (Swann et al. 2008) and in a xenografted glioma mouse model (Villeneuve et al. 2005, Nakagawa et al. 2007). In addition, TNF-α overexpression in cancer cells has shown long-term tumor growth suppression and TNF-α delivery to tumor sites promotes anti-tumor effects (Josephs et al. 2018). On the other hand, TNF stimulates proliferation, survival, migration and angiogenesis in most cancer cells that are resistant to TNF-induced toxicity, resulting in tumor promotion via activating NF-κB (Suzukawa et al. 2002, Wang and Lin 2008).

Aberrant NF-κB regulation has been observed in many cancers (Taniguchi and Karin 2018). In both tumor and inflammatory cells, NF-κB is activated downstream of the TLR pathway and of the inflammatory cytokines TNF-α and IL-1β (Hoesel and Schmid 2013). In addition, NF-κB activation can be the result of cell-autonomous genetic alterations (amplifications, mutations or deletions) in cancer cells (Colotta et al. 2009). Many different types of human cancers have misregulated NF-κB; that is, NF-κB is constitutively active (Pires et al. 2018, Taniguchi and Karin 2018, Brücher et al. 2019). NF-κB activation has been shown to upregulate the expression of cyclin D1 and cyclin-dependent kinase 2 (CDK2), which drives cell cycle progression and causes uncontrolled cell proliferation (Ledoux and Perkins 2014, Park and Hong 2016, Taniguchi and Karin 2018). The activation of NF-κB is strongly linked to the inhibition of apoptosis in cancer cells, likely due to the ability to regulate expression of anti-apoptotic genes, such as *TRAF1/TRAF2*, cellular inhibitor of apoptosis 1/2 (*c-IAP1/2*), *Bcl-2* and *XIAP* (Figure 2) (Lu et al. 2007, Gyrd-Hansen and Meier 2010, Park and Hong 2016). Additionally, NF-κB induces a number of cytokines that may contribute to tumor-promoting inflammation, such as TNF-α, IL-1β, IL-6, IL-8, monocyte chemoattractant protein-1 (MCP1) and cyclooxygenase 2 (COX2) (Yeh et al. 2013, Taniguchi and Karin 2018). In most cases NF-κB plays a role as a tumor promoter, especially in the chronic inflammation related cancers, although many studies have shown that inhibition of NF-κB reduces tumor initiation and progression (Xia et al. 2014, Brücher et al. 2019).

A major effector molecule of NF-κB activation is IL-6, a multi-functional cytokine with growth-promoting and anti-apoptotic activity, being also linked to the activation of STAT3 (Kitamura et al. 2017, Johnson et al. 2018). Depending of the cell type, IL-6 is able to act through several protein kinase pathways, such as mitogen activated protein kinase (MAPK) and phosphatidylinositol-triphosphate kinase (PI-3 kinase), whereas via the JAK pathway, IL-6 activates STAT1 and STAT3 (Jones and Jenkins 2018). Activation of IL-6/STAT3 signaling axis is an important event in cancer, which promotes tumorigenesis by regulating proliferation and survival signaling pathways in cancer cells. IL-6 acts directly on tumor cells to induce the expression of STAT3 target genes, which

encode proteins that drive tumor proliferation and/or survival (Johnson et al. 2018). Overexpression of IL-6 has been detected in several carcinomas, e.g. prostate cancer, lung cancer, ovarian cancer and many more (Culig and Puhr 2012, Chang et al. 2013, Maccio and Madeddu 2013, Kumari et al. 2016, Johnson et al. 2018). In addition to inhibition of apoptosis and regulation of proliferation, IL-6 also fine-tunes other cancer hallmarks, like promotion of survival, angiogenesis and invasiveness, and regulates cancer cell metabolism (Kumari et al. 2016). Since IL-6/STAT3 pathway is hyper activated in cancer, therapies that target individual nodes in this pathway are beneficial as they inhibit tumor cell growth and stimulate antitumor immunity (Johnson et al. 2018).

1.1.4 Lung cancer

Lung cancer is one of the most frequent malignant tumors and the leading cause of cancer deaths worldwide, accounting for about 13% of all new cancer diagnoses and 24% of all cancer deaths (Siegel et al. 2019). In Europe, it is the third most common form of cancer, following breast cancer and colorectal cancer (Ferlay et al. 2018). The most common cause of lung cancer is tobacco smoking, however, it is estimated that 10–20% of all patients diagnosed with lung cancer have never been smokers (de Groot et al. 2018). Lung cancer can be divided into two main types, non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) (Griffin and Ramirez 2017). NSCLC includes 3 histological types (adenocarcinoma (AC), squamous cell carcinoma (SCC) and large cell carcinoma) and these can be further divided into various subtypes (Griffin and Ramirez 2017). NSCLC accounts for ~ 85% of lung cancers, with lung adenocarcinoma (AC) being the most common subtype (Marshall et al. 2016, Inamura 2017).

Genomic aberrations in lung AC have been well characterized and include mutations in epidermal growth factor receptor (*EGFR*), v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*), v-Raf murine sarcoma viral oncogene homolog B *(BRAF)*, rearrangements in anaplastic lymphoma receptor tyrosine kinase (*ALK*) and c-ros oncogene 1, receptor tyrosine kinase (*ROS1)* and alterations in mesenchymal-epithelial transition factor (*MET*) (Skoulidis and Heymach 2019). They all are widely recognized as driver mutations that represent and characterize the biology, epidemiology, prognosis and therapeutic susceptibility of lung AC and are routinely assessed in the clinic to offer targeted therapy for lung AC patients (Cardarella and Johnson 2013, Kris et al. 2014, Barlesi et al. 2016, Zugazagoitia et al. 2017). Unfortunately, lung AC and lung SCC have distinct driver mutation profiles. Common alterations in lung SCC are mutations in discoidin domain receptor tyrosine kinase 2 (*DDR2*), amplification in fibroblast growth factor 1 (*FGFR1*) and phosphoinositide-3-kinase, catalytic, alpha poly-peptide (*PIK3CA*) (Campbell et al. 2016, Tan et al. 2016). Development of targeted therapies has been less rapid for lung SCC compared to lung AC. For lung SCC, immunotherapy is showing promising results, as an anti-programmed

death protein 1 (PD-1) agent Nivolumab has been approved by the Food and Drug Administration (FDA) for lung SCC (Carbone et al. 2017).

Inactivation of tumor suppressor genes also plays an important role in the development of lung cancer. Accordingly, mutations in tumor suppressor genes, including neurofibromin 1 (*NF1),* retinoblastoma 1 (*RB1),* cyclin dependent kinase inhibitor 2A (*CDKN2A),* phosphatase and tensin homolog (*PTEN*) and tumor protein p53 (*TP53)* have been of use in molecular profiling of lung AC and SCC (The Cancer Genome Atlas Research 2014, Campbell et al. 2016, Tan et al. 2016).

1.1.5 Basonuclins

Human basonuclin 1 (BNC1) and basonuclin 2 (BNC2) are evolutionarily conserved C2H2 zinc finger proteins (Romano et al. 2004) with remarkable amino acid sequence conservation within their functional domains across species as distant as the zebrafish, chicken and mammals (Vanhoutteghem and Djian 2004). A comparison of mouse and human BNC2 encoding regions shows 91% identity at the nucleotide level and 97.2% identity at the amino acid level (Vanhoutteghem and Djian 2004). Corresponding values for BNC1 are 87% and 88%, respectively (Matsuzaki et al. 1997).

Human *BNC1* is located on chromosome 15 and was first identified as a gene highly expressed in skin keratinocytes, associated with proliferation (Tseng 1998, Vanhoutteghem et al. 2011). *BNC1* expression has been shown also in corneal epithelium and in reproductive germ cells (Mahoney et al. 1998, Tseng et al. 1999, Vanhoutteghem et al. 2011). BNC1 may function as transcription regulator affecting RNA polymerase I and II dependent gene expression (Zhang et al. 2007, Boldrup et al. 2012). It also has an essential role in oogenesis and spermato-genesis (Ma et al. 2006, Zhang et al. 2012).

Numerous studies have shown abberrant expression of *BNC1* in cancerous tissues, which may contribute to tumor progression. *BNC1* has been shown to be silenced by promoter methylation in a wide variety of tumors, including pancreatic cancer, renal cell carcinoma, lung cancer (Shames et al. 2006, Morris et al. 2010, Yi et al. 2013, Pangeni et al. 2015) and it is downregulated in hepatocellular carcinoma (HCC) cell lines and primary HCC tissues (Wu et al. 2016). In contrast, in breast cancer cells with increased invasive and metastatic capacity (Guo et al. 2011) and in squamous cell carcinoma of head and neck (Boldrup et al. 2012), *BNC1* expression is elevated.

Human *BNC2*, located on chromosome region 9p22, was discovered independently by two groups during searches of the EST databases with *BNC1* cDNA sequence (Romano et al. 2004, Vanhoutteghem and Djian 2004). Although the overall amino acid sequence of BNC2 differs extensively from its paralog BNC1*,* BNC2 possesses all the characteristic features of BNC1 – a serine-rich region, nuclear localization signal (NLS) and three pairs of distinct C2H2 zinc fingers (Vanhoutteghem and Djian 2004). Since it has been shown that BNC2 colocalizes with serine/arginine-rich splicing factor (SRSF2/SC35) in human

primary keratinocytes, its presumed function was associated with mRNA splicing or other forms of mRNA processing (Vanhoutteghem and Djian 2006). However, several studies have suggested that it might also act as a transcription regulator (Romano et al. 2004, Vanhoutteghem and Djian 2004, Vanhoutteghem and Djian 2006).

The *BNC2* mRNA is present in the same tissues where *BNC1* is highly expressed – the epidermis, the testis and the ovary (Romano et al. 2004, Vanhoutteghem and Djian 2004). In addition, *BNC2* mRNA is abundantly expressed in the kidneys, lungs, uterus and intestine (Romano et al. 2004, Vanhoutteghem and Djian 2004, Bhoj et al. 2011). Beyond its high expression in the skin, *BNC2* is also expressed in human primary keratinocytes and other epithelial cells, like immortalized human keratinocyte cell line HaCaT, human cervical cancer cells (HeLa) and embryonal kidney cells (HEK293) (Romano et al. 2004).

Several studies have shown aberrant expression of *BNC2* and its locus 9p22 associations in cancer, however, little is known about its function in tumor progression. Genome wide studies have identified significant associations between the 9p22 locus and cancer risk. Genetic variations in this region have been associated with skin cancer risk (Jacobs et al. 2015, Asgari et al. 2016, Chahal et al. 2016) and susceptibility to ovarian cancer (Song et al. 2009, Goode et al. 2010, Winham et al. 2014, Buckley et al. 2019). The decreased expresssion of *BNC2* mRNA has been detected in Barrett's esophagus tumor tissues (Akagi et al. 2009), HCC cell lines and primary HCC samples (Wu et al. 2016) and ovarian carcinoma and ovarian cancer cell lines (Goode et al. 2010, Cesaratto et al. 2016). Stable expression of *BNC2* resulted in the growth arrest of esophaegal adenocarcinoma cells (Akagi et al. 2009), suggesting its role as a tumor suppressor.

1.2 microRNAs

microRNAs (miRNAs) are known to be expressed in all eukaryotic multicellular organisms and by large DNA viruses and they are often conserved between multiple species (Shukla et al. 2011). miRNAs are short, 19 to 24 nucleotides long single-stranded RNAs that post-transcriptionally regulate gene expression (Ha and Kim 2014). miRNAs are generated in the nucleus as long primary transcripts (pri-miRNAs), which are processed by the Drosha-DiGeorge syndrome critical region 8 (DGCR8) complex resulting in precursor miRNAs (pre-miRNAs). The latter are next transported into the cytoplasm by a complex containing the Exportin 5 protein and GTP-binding nuclear protein, Ran-GTP (Yi et al. 2003, O'Connell et al. 2010, Ha and Kim 2014). In the cytoplasm, Dicer enzyme together with transactivator-responsive RNA-binding protein (TRBP) processes pre-miRNA, which involves the removal of the terminal loop, resulting in a mature miRNA duplex (Zhang et al. 2004). The directionality of the miRNA strand determines the name of the mature miRNA form. The 5p strand arises from

the 5' end of the pre-miRNA hairpin, while the 3p strand originates from the 3' end (O'Brien et al. 2018). Both strands derived from mature miRNA duplex can be loaded into the RNA-induced silencing complex (RISC). Processing of the miRNA duplex is mediated by the Argonaute (AGO) family of the proteins, in conjunction with several cofactors (Rupaimoole and Slack 2017). miRNAs incorporated in the RISC complex normally bind to their target mRNAs via partial complementarity to mRNA 3'UTR, using their "seed" sequence located at the first 2 to 8 nucleotides from the miRNA 5' end. The seed sequence defines miRNA families and is important for proper target recognition. miRNA binding consequently leads to the inhibition of target mRNA translation and ultimately to the reduction in protein expression levels (Bartel 2009, Kim et al. 2009). The canonical pathway of miRNA processing and function is shown in Figure 3.
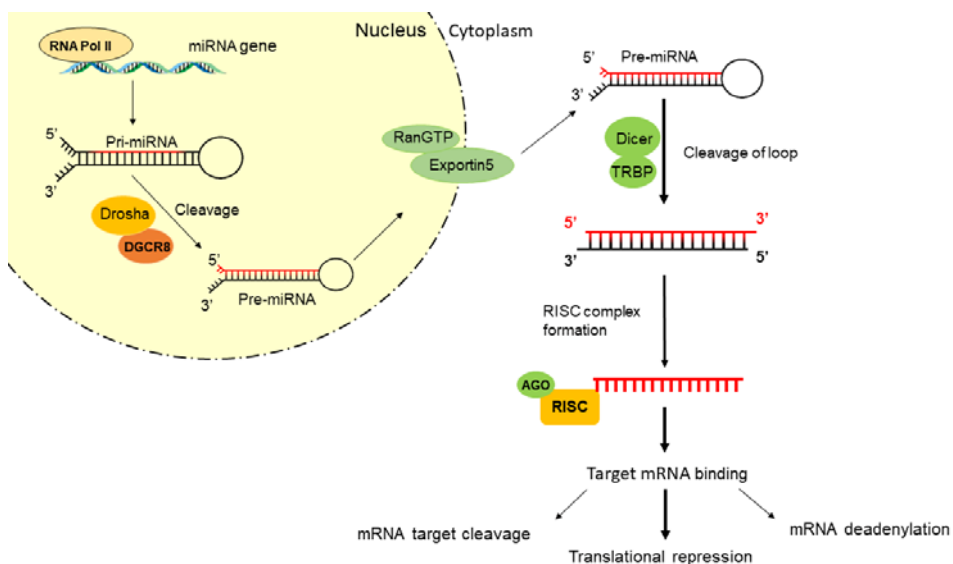
Figure 3. The miRNA biogenesis and function. The biogenesis of miRNAs starts with the transcription of pri-miRNAs by RNA polymerase II followed by cleavage of the pri-miRNA by the Drosha-DiGeorge syndrome critical region (DGCR) 8 complex in the nucleus. The resulting hairpin pre-miRNA is exported to the cytoplasm by Exportin 5 and GTP-binding nuclear protein, Ran-GTP (Exp5-RanGTP) complex. In the cytoplasm, Dicer in complex with transactivator-responsive RNA-binding protein (TRBP) cleaves the pre-miRNA hairpin to its mature length. In the next step, miRNA duplex is incorporated into the RNA-induced silencing complex (RISC) and subsequent processing is mediated by the Argonaut (AGO) family of proteins. Following unwinding and strand selection, the mature miRNA is capable of target recognition. Binding of the mature miRNA to RISC leads to the targeting of mRNAs containing complementary sites and results in translational repression or mRNA deadenylation and degradation. Figure is prepared according to the information of Rupaimoole and Slack (Rupaimoole and Slack 2017).

The functional importance of miRNAs is highlighted by their unique capability to simultaneously regulate many genes, which often are from the same pathway and thereby miRNAs affect a whole network of biological processes (Sozzi et al. 2011). Altogether, miRNAs influence various biological processes, such as cell proliferation, migration and apoptosis (Zhang et al. 2013), immunity (Taganov et al. 2006, O'Connell et al. 2010), control of cell cycle and cell death (Ng et al. 2012), intracellular signaling (Zhang et al. 2012), cellular metabolism (Rayner et al. 2011) and have been shown to act as crucial players during normal development (Ivey and Srivastava 2015).

1.2.1 miRNA expression is altered in diseases

As discoveries of novel human miRNAs are emerging, the research focus has been shifting towards functional characterization of miRNAs in the context of human diseases. miRNA expression patterns are tissue specific (Landgraf et al. 2007, Guo et al. 2014, Ludwig et al. 2016) and in many cases define the physiological state of the cell. As certain miRNA expression patterns could be disease-specific, miRNAs also have been suggested to be valuable diagnostic and prognostic biomarkers (Faruq and Vecchione 2015). In addition to cells and tissues, miRNAs can be found in the extracellular space, either in blood serum or other body fluids, such as saliva, urine and breast milk. In a particular disease, some miRNAs are over-expressed and some are underrepresented, giving rise to a signature miRNA pattern.

In case of tumors, miRNA expression profiles are very potential to classify different tumors in order to assist cancer diagnosis and predict survival (Berindan-Neagoe et al. 2014, Jarry et al. 2014, Tong et al. 2015). Association of miRNAs with cancer can have either diagnostic, prognostic or predictive value. For example, a three-miRNA signature (miR-145, miR-200c, and miR-218-1) predicts survival in cervical cancer (Liang et al. 2017) and a 18 miRNA pattern strongly correlates with lung AC and is associated with survival time (Yerukala Sathipati and Ho 2017). A set of twenty-two diagnostic miRNAs (17 upregulated and 5 downregulated) and 10 prognostic miRNAs were identified as significantly differentially expressed in pancreatic cancer (Calatayud et al. 2017). Circulating miRNAs are another source of cancer biomarkers. An eight-miRNA signature panel (miR-21-5p, miR-28-3p, miR-142-3p, miR-186-5p, miR-191-5p, miR-197-3p, miR-425-5p and miR-590-5p) analysis in plasma samples was identified to differentiate head and neck squamous cell carcinoma (HNSCC) patients and healthy donors (Summerer et al. 2015). Increased serum levels of various miRNAs have been linked to different human cancers, for instance, miR-141 to prostate cancer, miR-25 and miR-223 to lung cancer, miR-21, miR-92, miR-93, miR-126 and miR-29a to ovarian cancer and miR-210, miR-21, miR-155 and miR-196a to pancreatic cancer (Hamam et al. 2017).

The potential of circulating miRNAs as biomarkers of diseases has mainly been demonstrated for various types of cancer. However, recent attention has been turned to the use of circulating miRNAs as diagnostic/prognostic biomarkers

also for other conditions, including infectious diseases, such as human tuberculosis caused by infection of *Mycobacterium tuberculosis*, sepsis, viral hepatitis and for several other diseases (Correia et al. 2017), as well as for inflammatory diseases, such as asthma, inflammatory bowel disease, and rheumatoid arthritis (Mi et al. 2013). Deregulation of miRNAs has been shown also in asthmatic patients. Three miRNAs (miR-629-3p, miR-223-3p, miR-142-3p) were found to be significantly upregulated in asthmatics compared to healthy control subjects (Maes et al. 2016) and a five-miRNA signature distinguishes asthma patients from healthy controls (Milger et al. 2017).

1.2.2 miRNAs in tumor microenvironment

Tumors are characterized by the presence of various genetic and epigenetic alterations (Hanahan and Weinberg 2011, Shen and Laird 2013), which lead to deregulation of a number of miRNAs and gene expression in general (Rupaimoole et al. 2016).

Abnormal regulation of miRNA expression has been shown in nearly all human cancers to interfere with important cellular processes, such as differentiation, proliferation and apoptosis, resulting in the loss of homoeostasis (Croce 2009). Analogous to protein coding genes, miRNAs can act as tumor suppressors ("TSG-miR"), inhibiting tumor development by suppression of oncogenes and/or genes involved in the cell cycle regulation. Other tumor-associated miRNAs act as oncogenes ("onco-miRs") that promote the development of tumors by negatively regulating tumor suppressor genes and/or genes that control cell differentiation and apoptosis (Zhang et al. 2007). Onco-miRs are typically overexpressed in cancer cells, while TSG-miRs are downregulated as compared to related non-neoplastic cell types (Pramanik et al. 2011).

Examples of TSG-miRNAs are let-7 family miRNAs, which were the first identified miRNA in humans (Pasquinelli et al. 2000) and that are widely viewed as tumor suppressor miRNAs (Zhang et al. 2007). Consistently, the expression of *let-7* family members is downregulated in many cancer types as compared to normal tissue (Boyerinas et al. 2010). On the other hand, the overexpression of *let-7* directly inhibits key cell cycle proto-oncogenes in human cancer cells, e.g. rat sarcoma oncogene (*RAS*), cell division cycle 25a (*CDC25a*), *CDK6* and cyclin D, thus controlling cell proliferation by suppressing genes in pathways promoting the G1 to S transition (Johnson et al. 2007).

Another well-studied TSG-miRNA is miR-34a. It is highly expressed in normal tissues and commonly repressed in many carcinomas, e.g. breast cancer (Li et al. 2013), NSCLC (Garofalo et al. 2013) and prostate cancer (Duan et al. 2015), and it is suggested to have a therapeutic effect in cancer (Li et al. 2014, Adams et al. 2016). miR-34a strongly influences the tumor suppressive regulatory network controlled by p53, encoded by the *TP53* gene. Several studies have shown that the expression of *miR-34a* is upregulated by p53 (Tarasov et al. 2007, Navarro and Lieberman 2015). The genes regulated by miR-34a include

several post-translational inhibitors of p53, a strong p53 transactivation inhibitor *MDM4* and *TP53* itself. In line with this, upregulation of *miR-34a* results in cell cycle arrest and apoptosis (Chang et al. 2007, He et al. 2007). In addition, miR-34a directly inhibits the expression of another transcription factor, anti-apoptotic silent information regulator 1 (SIRT1) (Li et al. 2013) and affects the expression of cell cycle proteins, such as CDK4 and CDK6, as well the anti-apoptotic protein Bcl-2 and metastasis-related proteins, such as MET, Notch, v-myc avian myelocytomatosis viral oncogene homolog (MYC) and AXL receptor tyrosine kinase (AXL) (Li et al. 2013, Misso et al. 2014). As a result, miR-34a carries characteristic tumor suppression effects, such as cell proliferation inhibition, cell cycle arrest, and induction of senescence and apoptosis (Sun et al. 2008, Yamakuchi et al. 2008, Hermeking 2009, Ghawanmeh et al. 2011, Liu et al. 2011, Sotillo et al. 2011, Zauli et al. 2011, Heinemann et al. 2012). Moreover, recent studies have demonstrated that in acute myeloid leukemia (AML) and in NSCLC, miR-34a also downregulates the programmed cell death protein 1 ligand (PD-L1) (Wang et al. 2015, Cortez et al. 2016).

Comparing cancer to adjacent normal lung tissue, oncogenic miRNAs are defined by a higher expression in the malignant lung tissues. Among oncogenic miRNAs, *miR-21* is one of the most represented, being over-expressed in various types of solid tumors (Volinia et al. 2006). Overexpressed *miR-21* downregulates the expression of tumor suppressor gene *PTEN* (Zhang et al. 2010), pro-apoptotic gene programmed cell death 4 (*PDCD4*) (Asangani et al. 2007, Zhu et al. 2008, Bhatti et al. 2010), and tumor suppressor gene tropomyosin 1 (*TPM1*) (Zhu et al. 2008), thereby promoting cell proliferation and migration, and inhibiting apoptosis.

1.2.3 miRNAs in inflammation

The expression of numerous miRNAs is altered during inflammation and many of them influence inflammatory responses. For example, several miRNAs have been shown to be affected and themselves influence the activation of the NF-κB pathway. Among others, miR-146a is one of the most intensively studied inflammatory response miRNA, which is upregulated by NF-κB and is known to act as a negative regulator of inflammation in numerous cell types (O'Connell et al. 2007, Rebane and Akdis 2013). For example, in human lung alveolar epithelial tumor A549 cells, IL-1β induces the expression of *miR-146a*, which then negatively regulates the release of chemokines IL-8 and C-C motif chemokine ligand 5 (CCL5) (Perry et al. 2008). Among miR-146a targets, the best characterized are its direct targets IRAK1, TRAF6 and caspase recruitment domain family member 10 (CARD10), which are all upstream activators of NF-κB (Taganov et al. 2006, Crone et al. 2012).

The capacity of miR-146a to be activated and to regulate a central inflammatory pathway proposes that miR-146a has anti-inflammatory function also in several diseases, including chronic inflammatory skin diseases. Accordingly, *miR-146a* expression is elevated in keratinocytes and in the skin of psoriasis and

atopic dermatitis (AD) patients (Sonkoly et al. 2007, Rebane et al. 2014). Moreover, the lack of miR-146a leads to a stronger skin inflammation in a mouse models of AD and psoriasis (Meisgen et al. 2014, Rebane et al. 2014, Srivastava et al. 2017). Interestingly, although miR-146a inhibits the expression of many pro-inflammatory cytokines and chemokines, including CCL5, CCL8, CCL20 and IL-8 in human primary keratinocytes, its upregulation in diseased skin is not enough to suppress inflammation in psoriasis and AD (Hermann et al. 2017, Srivastava et al. 2017). This indicates that overexpression or delivery of miR-146a into the inflamed skin may have a therapeutic potential in the suppression of inflammation.

Besides miR-146a, numerous other miRNAs target the NF-κB pathway. An interesting example is miR-155 which, in contrast to miR-146a, has pro-inflammatory characteristics. miR-155 targets and downregulates the negative regulators, such as SH-2 containing inositol 5' polyphosphatase (SHIP1) and SOCS1, which leads to increased activation of AKT serine/threonine kinase (Androulidaki et al. 2009, O'Connell et al. 2009). In addition, miR-155 represses directly the expression of *Bcl-6*, a transcription factor that attenuates pro-inflammatory NF-κB signaling (Nazari-Jahantigh et al. 2012).

1.3 Development of delivery systems for nucleic acid therapeutics

As our understanding of genetics, gene expression regulation and molecular pathogenesis has improved, the development of nucleic acid (NA) therapeutics has received more attention as a strategy to modulate the cellular machinery by controlling the expression levels of functional proteins. Therefore, the field of cellular delivery systems for NAs has also gained an increased attention and undergone a rapid progress over the last decade.

Various NA-based molecules have been studied for their potential as therapeutic molecules, including plasmid DNA (pDNA), antisense oligonucleotides (ASO), small hairpin RNAs (shRNAs), small interfering RNAs (siRNAs), miRNAs and mRNA (Stewart et al. 2018, Kowalski et al. 2019). As all these molecules are significantly bigger than classical small molecule drugs and carry high negative charge, their cellular uptake is marginal (Lam et al. 2015).

For delivery of NAs, two main strategies, viral and non-viral methods, are used. Viral vectors, such as retrovirus, adenovirus, adeno-associated virus and lentivirus have high efficiency and specificity in delivering genetic materials to the cells and have recently made huge steps towards the clinical applications for treatment of many genetic disorders (Naldini 2015). Viral vectors have been implemented in clinical trials for decades (Thomas et al. 2003, Kay 2011), however, viruses always retain some of their inherent hazards, including potential immunogenicity, tumorigenicity, limited cargo-carrying capacity, laborious production and purification, etc. Importantly, viral vectors are not universally

applicable for all NA-based molecules. For example, they are not compatible with the delivery of short synthetic oligonucleotides (Lehto et al. 2012). Therefore, in the context of oligonucleotide delivery, focus has always been on the development of non-viral delivery strategies. Non-viral delivery systems comprise both the physical and chemical strategies. Physical methods involve induction of transient mechanical disruption of cell membrane for localized delivery of naked nucleic acid molecules. Current physical delivery methods include direct penetration, permeabilization and membrane disruption. Direct penetration strategies deliver exogenous molecules directly into the cell cytoplasm and most prevalent examples are microinjection and using ballistic particles or nanoneedles (Slivac et al. 2017, Stewart et al. 2018). Mechanical, electrical, optical, thermal and chemical permeabilization strategies make the cell transiently permeable to cargo from the extracellular solution (Stewart et al. 2018). Electroporation is a widely used membrane disruption method in which electrical field is applied to cells in order to transiently disrupt the plasma membrane by creating pores through which molecules can enter the cells (Slivac et al. 2017).

Chemical methods include a great variety of delivery vehicles, such as metallic nanoparticles (Sokolova and Epple 2008), cationic polysaccharides (Saranya et al. 2011), polymers (Laga et al. 2012), polymersomes (Dennis and Fariyal 2006), liposomes (Elouahabi and Ruysschaert 2005, Daraee et al. 2016) and cell-penetrating peptides (Lehto et al. 2012, Margus et al. 2012, Nayerossadat et al. 2012, Wang et al. 2014, Kristensen et al. 2016). In case of chemical methods, NA complexes with polymers can be self-formed in the solution, or the carrier directly conjugated with NAs. The use of bioconjugation as a method of delivering NA to cells involves coupling of NA with biomolecules to form conjugates capable of specifically binding to receptors on the cell membrane (Chernikov et al. 2019). One of the most successful examples of this strategy is the use of N-acetylgalactosamine (GalNAc), which allows receptor-mediated endocytosis of siRNAs via the asialoglycoprotein receptor (ASGPR) expressed on the surface of hepatocytes (Nair et al. 2014, Brown et al. 2019).

1.3.1 Liposomal delivery systems

Liposomes have been extensively used vehicles for the delivery of nucleic acids ever since their ability to transport the preproinsulin gene to the liver was demonstrated (Soriano et al. 1983). Liposomes are vesicles with an aqueous milieu in lumen enclosed in a phospholipid bilayer. Liposomes usually self-form from lipids and NAs in the solution. In the liposome, NAs in lumen are protected from enzymatic degradation and are delivered into the cells because of the capacity of liposome to interact with negatively charged cell membrane (Gao et al. 2011). The cellular delivery of these complexes occurs via endocytosis followed by endosomal escape of the cargo into the cytoplasm (Sahay et al. 2010).

Three types of liposomes can be distinguished based on the charge: cationic, anionic, and neutral. Cationic liposomes are most commonly used as non-viral

delivery systems due to their unique characteristics, including easy production, high affinity for the cell membrane, and absence of or low pathogenicity and immunogenicity (Yang 2015). Many cationic liposomes for NA delivery are commercially available and have been in use in several studies, for example, Lipofectamine® RNAi-Max and Lipofectamine 2000/3000 (LF2000/3000) by Thermo Fisher Scientific (Zhao et al. 2008, Peng et al. 2013, Jensen et al. 2014), SilentFect™ by Bio-Rad (Zhao et al. 2009), DharmaFect® by Thermo Fisher Scientific (Sundaram et al. 2013, Jensen et al. 2014) and SiPORT™ also by Thermo Fisher Scientific (Šahmatova et al. 2016). Although cationic lipid-based delivery systems offer several advantages for NA delivery in vitro, main critical disadvantages of the liposome based delivery system are the short half-life (Akbarzadeh et al. 2013) and the fact that high transfection efficiency with lipofection typically correlates widely with cell toxicity (Andaloussi et al. 2011, Lehto et al. 2011, Ozpolat et al. 2014).

1.3.2 Cell-penetrating peptides

The identification of short proteins with the ability to translocate across the cell membrane was first reported by two independent groups in the late 1980s. The Trans-Activator of Transcription (Tat) protein of the human immunodeficiency virus was the first identified cell-penetrating protein (CPP) able to efficiently enter tissue-cultured cells and promote the viral gene expression (Frankel and Pabo 1988, Green and Loewenstein 1988). In subsequent years, several CPPs were identified, for example, a fragment of homeotic protein of *Drosophila melanogaster* Antennapedia (pAntp) was shown to enter nerve cells and regulate neural morphogenesis (Joliot et al. 1991) and a herpes virus structural protein vp22 showed a potential in protein delivery (Elliott and O'Hare 1997). The peptide sequences derived from these proteins that possess the cell-penetrating activity are nowadays widely used and are covalently attached to cargoes in most cases.

Since the discovery of CPPs, the field has rapidly expanded and numerous new CPPs have been designed for cellular transport. Altogether, over 1850 peptides have been described according to CPP database http://crdd.osdd.net/ raghava/cppsite/ (Agrawal et al. 2016). CPPs are relatively short peptides, generally less than 30 amino acids in length, that have the capacity to ubiquitously cross cellular membranes, with very limited toxicity and without the necessity of a recognition by specific receptors (Lindgren et al. 2000). They are typically classified based on their chemical and physical characteristics as polycationic, amphipathic, or hydrophobic (Pooga and Langel 2015). According to their origin (Table 1), we can distinguish three main classes of CPPs: peptides derived from proteins (e.g Tat peptide), chimeric peptides (e.g transportan, PepFects) that are formed by the fusion of two natural sequences, and synthetic CPPs (e.g oligoarginine, CADY, WRAP) which are rationally designed sequences usually based on structure-activity studies (Rothbard et al. 2000, Crombez et al. 2009, Bechara and Sagan 2013, Konate et al. 2019).

Table 1. Examples of delivery peptides

| Peptide	Sequence	Origin/ Modification	Reference
Protein derived CPPs			
Tat (47–57)	GRKKRRQRRRPPQC	HIV-1 Tat protein	(Vives et al. 1997)
Penetratin (pAntp)	RQIKIWFQNRRMKWKK	Antennapedia homeodomain of drosophila	(Derossi et al. 1994)
Chimeric CPPs			
Transportan	GWTLNSAGYLLGKINLKA LAALAKKIL	Fusion of neuropeptide galanin and wasp venom peptide	(Pooga et al. 1998)
TP10	AGYLLGKINLKALAALAK KIL-NH$_2$	Fusion of neuropeptide galanin and wasp venom peptide	(Soomets et al. 2000)
PepFect3	Stearyl-AGYLLGKINLKA LAALAKKIL-NH$_2$	Direct analog of TP10 with N-terminal fatty acid modification	(Mäe et al. 2009)
PepFect6	Stearyl-AGYLLG**K**INL KALAALAKKIL-NH$_2$	Modified PF3, by attachment of trifluoromethylquinolin e-based moieties via lysine tree in the Lys7 position	(Andaloussi et al. 2011)
PepFect14	Stearyl-AGYLLGKLLOOL AAAALOOLL-NH2	Modified PF3, by replacing some lysines and the isoleucines with ornithines and leucines	(Ezzat et al. 2011)
Synthetic CPPs			
Oligo-arginine	(R)$_n$; n=6–12	Based on Tat peptide	(Rothbard et al. 2000)
CADY	Ac-GLWRALWRLLRSL WRLLWRA-cysteamide	Derived from the chimerical peptide PPTG1	(Crombez et al. 2009)

Since the discovery of Tat peptide, many studies have aimed to apply CPPs in delivery of NAs into cells *in vitro* and *in vivo*. For siRNAs, non-covalent complexes of PepFects (PF) have been shown to be efficient as they activate different endocytic routes when delivering nucleic acids to cell interior (Arukuusk et al. 2013, Pae and Pooga 2014). PF is a common name for a series of peptides that were initially designed based on Transportan 10 (TP10) sequence (Soomets et al. 2000). TP10 was first modified by adding N-terminal stearic acid to improve non-covalent complex formation with oligonucleotide cargoes and increase the membrane interactions of the peptide (Mäe et al. 2009). The peptide was later named PepFect3 and was later used as a basis for further modifications and amino acid alterations resulting, among others, in PepFect6 (PF6) and PepFect14 (PF14) (Table 1).

1.3.3 Cellular uptake mechanism of CPP-NA nanocomplexes

Since the first studies describing the ability of a class of small peptides to translocate across biological membranes in a very efficient, nontoxic manner, CPPs have been used extensively as delivery systems for NAs. CPPs can transport a variety of covalently or non-covalently linked cargoes inside living cells both *in vitro* and *in vivo*. These cargoes include miRNA inhibitors (Lindberg et al. 2013), siRNAs (Tai and Gao 2017), ASOs (Pooga et al. 1998, Rogers et al. 2012), proteins (Mäe and Langel 2006, Lehto et al. 2012), pDNA (Veiman et al. 2013, Veiman et al. 2015), nanoparticles, peptides and liposomes (Gupta et al. 2005).

Uptake of most CPPs is considered to start with the interaction of the peptide with negatively charged plasma membrane components, followed by internalization via different endocytosis mechanisms depending on the features of the CPP, its associated cargo, the target cell type and the membrane lipid composition (Juks et al. 2015). Several different types of endocytic uptake for most CPPs have been reported, including macropinocytosis (Wadia et al. 2004, Tanaka et al. 2012), clathrin dependent pathway (Lundin et al. 2008) and caveolin-dependent endocytosis (Padari et al. 2005) (Figure 4). However, some CPPs can directly penetrate into cells independently from endocytosis and this has been suggested as the main cell entry mechanism for CADY peptide (Rydström et al. 2011) and for some arginine-rich peptides (Hirose et al. 2012). Limitation of these methods is the entrapment of CPP-NA complexes in endosomes after cellular uptake by endocytosis. To enhance endosomal escape of CPP-NA nanocomplexes, different chemical modifications have been introduced into known CPPs (Arukuusk et al. 2013). Several of these modifications have improved the endosomal escape of CPPs. For example, PepFects (PF6 and PF14) are Transportan 10 (TP10) analogues with N-terminal fatty acid moiety (Mäe et al. 2009) and further modifications in the backbone of CPPs. PF6 was developed by covalent attachment of trifluoromethylquinoline-based moieties via a lysine tree in the Lys_7 position in stearyl-TP10 and was proved to be a very potent delivery vector for siRNAs in different cell culture models and *in vivo* (Andaloussi et al. 2011,

Pärnaste et al. 2017). To generate PF14, changes in the amino acid sequence of TP10 were carried out, by replacing some lysines and the isoleucines with ornithines and leucines, respectively, adding an extra positive charge (Ezzat et al. 2011). PepFect14 peptide proved to be a very effective delivery vector for ASO, siRNAs, pDNA and mRNA (Veiman et al. 2013, Pärnaste et al. 2017, Ervin et al. 2019, van den Brand et al. 2019, van der Bent et al. 2019). Developing CPPs with increased endosomolytic properties is a necessary step towards achieving biological effects at low concentrations for future *in vivo* applications.
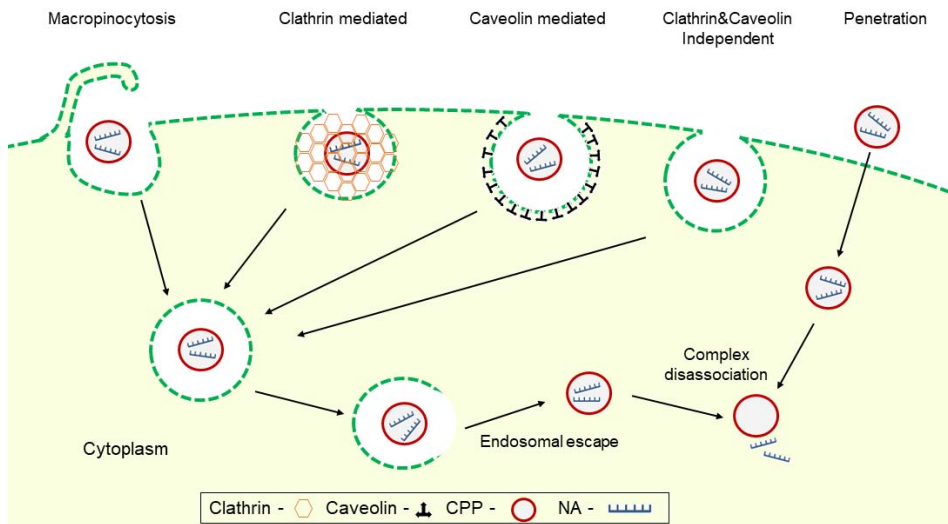
Figure 4. Overview of cellular uptake and intracellular trafficking of CPP-NA nanocomplexes via different endocytosis pathways. Multiple different cellular entry routes are available for CPP-NA nanocomplexes to cross cell plasma membrane via endocytosis mechanisms, including macropinocytosis, clathrin mediated, caveolin mediated, clathrin and caveolin independent endocytosis and penetration. Entered complexes are first entrapped in endosomes. For biological function, complexes need to escape endosomes and disassociate. Figure is modified according to the information of Margus and Cerrato (Margus et al. 2012, Cerrato et al. 2016).

1.3.4 RNA interference-based therapy

Since the discovery of RNA interference (RNAi), there has been a great interest in applying the knowledge of its basic essence for the treatment of diseases. RNAi is a cellular mechanism triggered by double-stranded RNA (dsRNA). In mammalian cells, it can be induced by either endogenous miRNAs and or synthetic siRNAs, which leads to the degradation of homologous RNAs (Mello and Conte Jr 2004). The ability of miRNAs to target multiple mRNAs that are altered in disease conditions makes these molecules promising candidates as

therapeutics (miRNA overexpression or use of miRNA mimics) or as a target of therapeutics (miRNA suppression by antagomiRs) (Rupaimoole and Slack 2017). Safe and effective delivery is the key to succeed in the development of oligonucleotide therapeutics as nonmodified oligonucleotides are often unstable and rapidly degraded by nucleases or may have unwanted side effects by activating the immune system (Khvorova and Watts 2017). Various chemical modifications are used to increase nucleic acid stability by preventing nuclease degradation and immunostimulation. Common modifications are made to the ribose (most often 2' position) or to the phosphodiester backbone. Most traditional modifications to ribose are 2′-*O*-methyl (2′-OMe), 2′-*O*-methoxyethyl (2′-MOE), 2′-fluoro (2′-F) and replacing ribose with locked nucleic acid (LNA). The aim of the modifications is to decrease immunogenicity and toxicity, increase RNA binding affinity, improve stability and bioavailability (Khvorova and Watts 2017, Smith and Zain 2019). Another common type of modifications are backbone modifications, such as phosphorothioate (PS), peptide nucleic acids (PNAs) or phosphorodiamidate morpholino (PMO), which provide protection from nucleases and increase binding affinity (Smith and Zain 2019).

The use of NA modifications and continuous improvements of *in vivo* delivery technologies have made miRNA/siRNA-based therapies feasible (Chakraborty et al. 2017, Rupaimoole and Slack 2017). One of the first miRNA mimics to enter a clinical study as a drug candidate was a liposome-formulated miR-34 mimic, MRX34 (Chakraborty et al. 2017). It was designed to deliver a mimic of tumor suppressor miR-34, which is under-expressed in a wide variety of tumors (Kreth et al. 2018). However, the study was halted due to the multiple severe immune-related adverse events (Kreth et al. 2018). More success has been achieved with anti-miRNA approach, among which Miravirsen, a locked nucleic acid (LNA) against miR-122 is in clinical trials for the treatment of hepatitis C virus (HCV) infection. A phase I clinical trial demonstrated that antagomiR-122 had dose-dependent pharmacodynamics and was well tolerated, showing only mild side effects in patients with HCV in phase II trial (Shah et al. 2016, Rupaimoole and Slack 2017, Kreth et al. 2018). In addition to Miravirsen, there are several other earlier phase clinical trials investigating new miRNA drug candidates. The company miRagen has three miRNA candidates in clinical development: phase I clinical trial of the drug MRG-110, a LNA modified antisense oligonucleotide to inhibit the function of miR-92 in case of wound healing and heart failure (Hanna et al. 2019); an active phase I study of MRG-201, which is a synthetic miRNA mimic of miR-29b for patients diagnosed with scleroderma; and a phase 2 trial for MRG-106 (Cobomarsen), a synthetic miRNA antagonist of miR-155 for patients diagnosed with cutaneous T cell lymphoma (CTCL) (Shah et al. 2016, Foss et al. 2017, Rupaimoole and Slack 2017).

Along with miRNA-based therapeutics, there are nearly 40 chemically-synthesized siRNA clinical trials and more candidates are on the way of preclinical study or waiting for being licensed for clinical study. Eight of chemically-synthesized siRNAs are investigated at phase III stage (Weng et al. 2019). Recently, the first siRNA-based therapeutic drug, ONPATTRO (Patisiran)

lipid complex injection, was approved by United States Food and Drug Administration for treatment of nerve damage caused by hereditary transthyretin amyloidosis (Adams et al. 2018, Weng et al. 2019, Yin and Rogge 2019). Multiple GalNAc-siRNA conjugate clinical trials, including two phase III trials, are currently underway by three biotech companies to treat wide variety of diseases, associated with hepatocytes and liver (Springer and Dowdy 2018).

2 AIMS OF THE STUDY

The general aim of the current doctoral thesis was to study the epithelial defense mechanisms and to investigate the potential of CPPs in therapeutic delivery of miRNAs in the same context.

The first part of the thesis was initiated by the discovery of a potential tumor suppressor candidate gene, *BNC2*, detected by our group during fine mapping of human chromosomal region 9p22. Not only the genomic location, but also the structural features, expression pattern and remarkable transcriptional flexibility of the *BNC2* gene evoked inspiration to study it further. To bring together the epithelial and immune cell types, inflammation and cancerous processes, our choice of a possible model system turned to respiratory tract. Accordingly, the first publication (Ref. I) summarizes our findings featuring BNC2 expression in lung cancer and function in human lung-derived cell culture models.

The research included in two other publications grew out from the need to evaluate the potential of miRNAs as possible candidates for drug development either in cancer or in targeting of inflammatory pathways in the epithelium. In addition, we hypothesized that selected CPPs suitable for delivery of siRNAs could be applicable also for delivery of miRNA mimics in cell cultures and *in vivo* and we aimed to test this approach in keratinocytes and skin (Ref. II) and in cancer cells (Ref. III).

The particular objectives of the current thesis were as follows:

1. To study the expression of a putative tumor suppressor gene *BNC2* in lung cancer and to investigate the effect of BNC2 on regulatory networks and viability of lung cancer cells.
2. To test the capacity of selected CPPs to deliver miR-146a mimics into human primary keratinocytes and mouse skin in a model of irritant contact dermatitis and to assess the efficiency of CPP-delivered miR-146a in the suppression of inflammatory responses in the same model systems.
3. To evaluate the efficacy of PF14 as a vehicle for delivering a miRNA analogue into cancer cells and to select a set of marker genes for rapid assessment of miR-34a-5p transfection efficiency.

3 MATERIALS AND METHODS

Research articles included in the current thesis contain data from patient tissue samples (Ref. I), cell culture experiments (Ref. I, II, III), mouse model of contact dermatitis (Ref. III) and array analysis data (Ref. I and III).

As patient material, lung squamous cell carcinoma (SCC) and corresponding adjacent non-tumor tissue samples were derived from 8 patients who had undergone curative surgery (lung resection) and histologically characterized by a clinical pathologist in Tartu University Hospital, Lung Clinic. The study was approved by the Research Ethic Committee of the University of Tartu and a written informed consent was obtained from all patients. These samples were used for quantification of BNC2 mRNA expression (Ref. I).

Overview of cell lines, primary cells, mouse strains, the used methods and the tools for data analysis are briefly presented in Table 2 and in detail in every corresponding method sections of References I, II and III. The experimental workflow for the mouse model of irritant contact dermatitis is presented in Figure 5. All animal experiments were approved by the Animal Ethics Committee at the Ministry of Agriculture Estonian Government. Mice were maintained in the animal facility at the Institute of Molecular and Cell Biology, University of Tartu, according to the institutional regulations.

Table 2. Cell-lines, mouse strains and methods

Cell line/ mouse strain	Methods	Paper
A549	RT-qPCR to analyze BNC2 mRNA expression	Ref. I
	Transient transfection of expression plasmid containing full-length human BNC2 coding sequence and corresponding empty plasmid pCMV-HA by electroporation	Ref. I
	Illumina HumanHT-12 v4 Expression Array for global gene expression analysis of transfected cells	Ref. I
	RT-qPCR for Illumina HumanHT-12 v4 Expression Array validation	Ref. I; Ref. III
	CellTiter-Glo Luminescent Cell Viability Assay for cell proliferation analysis	Ref. I
	Ingenuity Pathway Analysis (IPA) and g:Profiler tools for pathway and gene ontology enrichment analysis	Ref. I
	Transfection of miRNA mimics (miR-34a-5p and cel-miR-67 negative control) by PF14 and siPORT	Ref. III
BEAS-2B	RT-qPCR to analyze BNC2 mRNA expression	Ref. I

Cell line/ mouse strain	Methods	Paper
Human primary keratinocytes	Transfection of miRNA mimics (Dy547-labeled control miRNA, miR-146a and miRNA negative control) by PF6, PF14, CADY and siPORT	Ref. II
	Morphology analysis of miR-146a-CPP (PF6, PF14, CADY) nanocomplexes by negative staining transmission electron microscopy (TEM)	Ref. II
	RT-qPCR to analyze the effect of target gene expression after transfection with miR-146a mimics	Ref. II
	CellTiter-Glo Luminescent Cell Viability Assay for cell proliferation analysis	Ref. II
	Flow cytometry for apoptosis analysis	Ref. II
	Confocal microscopy for visualization of Dy547-labeled control miRNA mimic	Ref. II
C57BL/6	Mouse model of irritant contact dermatitis (ICD) (Figure 5)	Ref. II
	Immunofluorescence microscopy for visualization of Dy547-labeled control miRNA mimic	Ref. II
	Light microscopy for histology analysis of mouse ears	Ref. II
	RT-qPCR for analysis of miR-146a functionality and transfection efficacy	Ref. II
PPC-1	Transfection of miRNA mimics (miR-34a-5p and cel-miR-67 negative control) by PF14, LF2000, Lipofectamine RNAiMAX or siPORT	Ref. III
	Transfection of Dy547-labeled cel-miR-67 control mimic by PF14 and siPORT	Ref. III
	Fluorescent microscopy for visualization of Dy547-labeled cel-miR-67 control	Ref. III
	IlluminaHT-12 v4 Expression array for transcriptome profiling of transfected cells	Ref. III
	CellTiter-Glo Luminescent Cell Viability Assay for cell proliferation analysis	Ref. III
	RT-qPCR for Illumina HumanHT-12 v4 Expression Array validation and analysis of miR-34a functionality and transfection efficacy	Ref. III
	STRING tool for pathway enrichment and network analysis	Ref. III
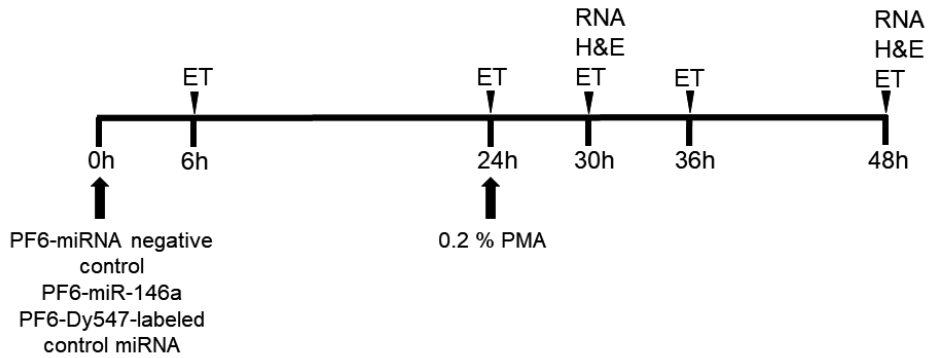
Figure 5 Experimental setup of ICD mouse model. Peptide (PF6) and miRNA mimics (miRNA negative control, miR-146a and Dy547-labeled control miRNA) nanocomplexes were injected subcutaneously into mice ears at time point 0 hour, 24 hours before application of 0.2% 12-myristate 13-acetate (PMA) in acetone on the mice ears, which induced irritant contact dermatitis (ICD). Ear thickness (ET) as indicator of inflammation was measured with digital caliper in three locations at 6, 24, 30, 36 and 48 h time-points. Tissue integrity by hematoxylin and eosin (H&E) staining and mRNA expression (RNA) of pro-inflammatory cytokines were analyzed at 30h and 48h time point. All of the mice were 8–10 week old wild-type C57BL/6. Further details can be found in the materials and the methods and results sections of Ref. II.

4 RESULTS AND DISCUSSION

4.1 BNC2 modulates the expression of IFN-regulated genes and acts as a tumor suppressor in lung cancer epithelial cells (Ref. I)

Lung cancer has remained the most common cancer in the world and is the leading cause of cancer-related deaths worldwide (Siegel et al. 2019). The average 5-year survival rate for lung cancer is still low, in spite of improvements in molecular diagnostics and targeted therapy (Siegel et al. 2015). Novel approaches and therapeutic targets are needed to control this disease.

Cancer is a consequence of mutations in the genes that control cell proliferation, differentiation and cellular homeostasis and these genes can be categorized into two classes: oncogenes and tumor suppressors. Overexpression of oncogenes and loss of tumor suppressors are the dominant driving factors for tumorigenesis. Hence, targeting oncogenes and tumor suppressors holds tremendous therapeutic potential for anti-cancer therapy (Guo et al. 2014). In the last decades, predominant cancer treatment options rely on designing inhibitors for the known oncogenic targets, such as MYC or RAS oncogenes and several growth factor receptors (Chen et al. 2018, Ferguson and Gray 2018, Ryan and Corcoran 2018). Despite the significance of oncogenes in the genesis of tumors, alterations that lead to the development of cancer tend to affect tumor suppressors even more often (Morris and Chan 2015).

Based on previous experiments conducted in our lab (during 1997–2003, unpublished data), aiming to describe human chromosome 9p22 genomic organization, we considered the newly discovered zinc finger encoding region therein to be interesting for further research. Presence of zinc fingers in the predicted protein was suggestive for function as a transcription factor and we planned expression experiments to reveal downstream targets and pathways. Independently, this gene was described in 2004 by two other groups (Romano et al. 2004, Vanhoutteghem and Djian 2004). Later on, BNC2 was shown to be downregulated in cancerous cells and tissues, including esophageal adeno-carcinoma cells, hepatocellular carcinoma cell lines and epithelial ovarian cancer cell lines (Akagi et al. 2009, Goode et al. 2010, Cesaratto et al. 2016, Wu et al. 2016). As lung cancer is continuously highly prevalent, our choice of a possible model system turned to respiratory tract, e. g. lung-derived cell lines and patient materials from lung cancer surgery specimens. Thus, we first assessed the expression of *BNC2* in association with lung cancer comparatively in two lung epithelial-derived cell lines – A549 as cancer cells, BEAS-2B as normal human bronchial epithelial cells. A significantly lower *BNC2* expression level was detected in the A549 cell line as compared to the BEAS-2B cell line (Ref. I, Figure 1A). We also tested whether *BNC2* expression is decreased in lung cancer tissue by analyzing eight pairs of matched SCC and adjacent non-tumor tissues. In accordance with the results in cell lines, SCC tissue samples showed decreased

BNC2 mRNA expression levels when compared to adjacent non-tumor tissue samples (Ref. I, Figure 1B).

One option for cancer therapy is to restore or reactivate tumor suppressor functions to normal level in order to inhibit cancer cell growth. Restoring or reactivating tumor suppressor function leads to the inhibition of proliferation of cancer cells and tumor growth and induction of cell-cycle arrest and/or apoptosis. For example, transfection of *PTEN* mRNA into prostate cancer cells *in vitro* and *in vivo* significantly inhibited tumor growth and induced apoptosis (Islam et al. 2018). Thus, as *BNC2* was downregulated in SCC and A549 cells, we examined whether *BNC2* overexpression could influence the proliferation rate and affect global gene expression pattern of the A549 cell line. We detected more than 20-fold increase in *BNC2* mRNA expression in A549 cells when transfected with BNC2-coding plasmid (Ref. I, Figure 2A), which in turn led to reduced proliferation rate of A549 cells (Ref. I, Figure 2B). Similar effect has been shown in esophageal adenocarcinoma cells, where stable expression of *BNC2* caused growth arrest of tumor cells (Akagi et al. 2009) indicating that our data are in line with previously published studies corroborating its role in tumor suppression. Furthermore, BNC2 has been described as a presumptive tumor suppressor gene also in glioblastoma and in urothelial carcinoma of the bladder (Nord et al. 2009, Beothe et al. 2015).

To analyze the impact of *BNC2* overexpression to global gene expression, we performed microarray data analysis followed by pathway analysis. Altogether, the expression of 152 genes (139 upregulated, 13 downregulated) was altered in response to *BNC2* overexpression, and of those, top 30 with the largest fold change are presented in heatmap (Ref. I, Figure 2C). Interestingly, the following pathway analysis of the expression change data for A549 cells transfected with BNC2 revealed interferon signaling as the most significantly influenced pathway (Ref. I, Figure 3). This was a novel and interesting finding, as previous studies had shown BNC2 ability to suppress the NF-κB basal activity in HEK293 cells (Li et al. 2011). Among interferon-regulated genes, our pathway analysis revealed the increased BNC2-dependent expression of several ISGs known to be involved in the regulation of tumor growth and development, such as *IRF7*, *XAF1* and *OAS* family genes (*OAS1*, *OAS2*, *OAS3* and *OASL*) (Ref. I, Figure 2C and 4). A subset of the latter (e.g. XAF1, IRF7 and OAS family) is known to promote apoptosis and thereby to inhibit cancer growth (Ning et al. 2011, Zhu et al. 2014, Choi et al. 2015).

For example, XAF1 has been proven to function as tumor suppressor in multiple cancers. It has been shown to inhibit cancer cell proliferation, induce apoptosis and inhibit tumor growth in hepatocellular carcinoma (Zhu et al. 2014), pancreatic cancer cell lines (Huang et al. 2010) and squamous cell lung cancer (Chen et al. 2011), by negatively regulating XIAP, which has the caspase-inhibiting and anti-apoptotic ability (Liston et al. 2001). Similarly, other genes upregulated by BNC2 have the capacity to inhibit cancer cell proliferation and induce apoptosis (*STAT*s, *IFN*-induced protein with tetratricopeptide repeats (*IFIT*)*1-3*, *ISG12a*, *IFITM3* and *OAS* family members) (Mullan et al. 2005,

Rosebeck and Leaman 2008, Stawowczyk et al. 2011, Chen et al. 2015, Zhang et al. 2017), further confirming that BNC2 has a role in the regulation of ISGs with anti-cancerous properties.

In the aggregate, our results show that BNC2 is downregulated in cancerous lung cell line A549 and SCC-derived surgical specimens. In addition, we show that overexpression of *BNC2* leads to reduced proliferation rate of A549 cells and upregulation of a set of interferon-regulated genes known to be implicated in the suppression of tumor growth and development. Together, our results and several previous studies in other cancer types suggest that BNC2 has characteristic features of a tumor suppressor protein. However, further studies are clearly needed to elucidate the role of BNC2 in suppression of cancerous processes *in vivo* and to evaluate the potential of BNC2 overexpression as possible treatment option.

4.2 miR-146a-PepFect nanocomplexes have an anti-inflammatory function in keratinocytes and in a mouse model of ICD (Ref. II)

The lack of efficient, safe, and specific methods for delivery of miRNA mimics in *in vivo* conditions is one of the main obstacles in the development of miRNA-based therapeutics (Kanasty et al. 2013). Another important question is whether the targeting or application of individual miRNAs, which often enable only a fine-tuning of expression level of their targets, is sufficient for treating diseases or alleviating disease-related conditions.

In the current study, we used the knowledge of miR-146a functions to test whether the selected CPPs support cellular delivery of miRNAs in keratinocyte culture and in case of skin inflammation *in vivo* in mice. When we started this study, miR-146a was known to have anti-inflammatory properties in numerous cell types and diseased conditions, including skin keratinocytes and AD (Meisgen et al. 2014, Rebane et al. 2014). Also, miR-146a direct targets, such as IRAK1, CARD10 and CCL5 (Crone et al. 2012, Rebane et al. 2014) as well as indirectly suppressed chemokines, including IL-8 and CCL20, were well defined. This made the effects of miR-146a mimics easy to test and suggested that application of miR-146a mimics could possibly have anti-inflammatory effect in case of skin inflammation *in vivo*. As delivery vehicle, we choose for our studies CPPs PF6, PF14 and CADY that had been previously shown to facilitate the delivery of siRNAs (Crombez et al. 2009, Andaloussi et al. 2011, Rydstrom et al. 2011, Ezzat et al. 2012).

Efficient delivery and cellular uptake by any endocytotic pathway is greatly dependent of the size and shape of the nanoparticles (Herd et al. 2013). The optimal size of the nanoparticles that efficiently enter the cells is in the range 30–70 nm in diameter (Chithrani et al. 2006). Earlier studies have demonstrated that PF6 and PF14 form non-covalent nanocomplexes with siRNAs with average diameter of about 100 nm at charge ratios optimal for transfection (Margus et al.

2016). Thus, in the current study, we first tested the capacity of selected CPPs to form nanocomplexes with miRNA mimics. We demonstrated that PF6 formed homogenous nanocomplexes with miR-146a in the diameter range of 30–55 nm (Ref. II, Figure 1A), while PF14-miR-146a nanocomplexes were morphologically more diverse (Ref. II, Figure 1A) and with CADY, majority of them were large agglomerates (Ref., Figure 1A).

Next, to monitor the efficiency of cellular uptake of nanocomplexes, human primary keratinocytes were transfected with Dy547-labelled miRNA mimics using either PF6, PF14 and CADY and analyzed by confocal microscopy. Our results show the strongest signal with PF6 and CADY (Ref. II, Figure 1B) and less intensity with PF14 at 24h time point (Ref. II, Figure 1B), indicating that all three tested CPPs are capable of delivering miRNA mimics into keratinocytes. Next goal was to evaluate whether delivered miR-146a mimics are released from the delivering complex and thus functional. For that, we transfected human primary keratinocytes using PF6, PF14 or CADY in unstimulated or inflammation mimicking conditions, where we stimulated cells with IFN-γ. IFN-γ was chosen, as we had previously observed strong activation of particular miR-146a-influenced genes, such as *CCL5* and *IL-8*, in keratinocytes in a similar experimental setting (Rebane et al. 2014). Indeed, an efficient suppression of miR-146a direct targets from the NF-κB pathway, CARD10 and IRAK1 (Ref. II, Figure 2A) and chemokines, IL-8 and CCL5 (Ref. II, Figure 2B) was observed with PF6-miR-146a nanocomplexes in unstimulated and IFN-γ-stimulated keratinocytes. In addition to PF6, suppression of direct targets and chemokines CCL5 and IL-8 in IFN-γ-stimulated keratinocytes was detected when we transfected cells with PF14-miR-146a nanocomplexes (Ref. II, Figure 2B). However, no effect of miR-146a on target genes and tested chemokines was observed, when CADY was used for the transfection (Ref. II, Figure 2A/B). This indicates that although CADY is efficient in delivery, there probably is no release of miRNA mimic from the complex. Efficient cellular uptake of nucleic acids in complex with CPPs in most cases occurs via endocytosis. However, as most of the cargo remains entrapped in the endosomes, the delivery efficacy depends greatly of the efficiency of endosomal escape (Arukuusk et al. 2013, Pärnaste et al. 2017).

Our CPP-miRNA nanocomplex delivery results are in line with previous studies demonstrating the successful delivery of siRNAs by PF6 and PF14. Accordingly, PF6/siRNA nanocomplexes have been shown to efficiently enter the cell, promote endosomal escape and result in robust RNAi responses without detectable toxicity in wide variety of cell types (Andaloussi et al. 2011). Similarly, PF14, has been shown to efficiently deliver splice correcting oligo (SCO)s (Ezzat et al. 2011) and siRNA (Ervin et al. 2019). Interestingly, some CPPs can mediate siRNA-delivery into cells by direct translocation, independent from endocytosis. This kind of entry has been suggested for the secondary amphipathic cell-penetrating peptide CADY (Crombez et al. 2009, Rydstrom et al. 2011). Our study proposes that cellular uptake, delivery and endosomal escape mechanisms of miRNA mimics are very similar to those of other short

oligonucleotides. However, there may be differences in miRNA transfection in case of peptides designed for endocytosis independent delivery.

Lipid-based delivery methods of nucleic acids are often accompanied by side effects, such as toxicity and inhibition of cell proliferation (Andaloussi et al. 2011, Suhorutsenko et al. 2011, Li and Rana 2014). To test whether the transfection of PF6- and PF14-based nanocomplexes with miRNA mimics affect the cell proliferation, we performed ATP-based viability assays. For comparison, we used lipid based commercial transfection reagent siPORT. As the results demonstrate, the transfection of miR-146a mimics with PF6 and PF14 affected the viability of primary keratinocytes significantly less than the lipid based transfection reagent siPORT in two different cell densities (Ref. II, Figure 3A).

As PF6-miRNA nanocomplexes were more homogenous as compared to PF14 (Ref. II, Figure 1A) and were very efficient in *in vitro* experiments (Ref. II, Figure 2), we next studied the capability of PF6-miRNA nanocomplexes to penetrate and to spread in the inflamed skin *in vivo*. For that, we performed subcutaneous injection of PF6-miRNA nanocomplexes into the mouse ears, followed by induction of irritant contact dermatitis (ICD) in some mice. We chose a mouse ear model of ICD because it is a straightforward, simple and easy skin inflammation model in response to a direct chemical agent (irritant). In this mouse model, the rapid inflammation is induced by the application of PMA (irritant) onto mouse ears, which causes the activation the NF-κB pathway, among other pathways, in the keratinocytes, leads to inflammation, activation of pro-inflammatory cytokines and increase in ear thickness (Kumari et al. 2014) and reduces the barrier function of tight junctions between the cells (Kirschner and Brandner 2012). Without PMA treatment, subcutaneously injected PF6-Dy547-labeled control miRNA nanocomplexes were detected as individual spots mostly in the dermis (Ref. II, Figure 4B). However, in the inflamed conditions, the PF6-Dy547-labeled miRNA complexes were visible throughout the entire cross-section of ear (Ref. II, Figure 4B). Furthermore, subcutaneous pre-injection of PF6-miR-146a nanocomplexes suppressed the ear-swelling response (Ref. II, Figure 4C) and reduced the expression of pro-inflammatory cytokines IL-6 and C-X-C Motif chemokine ligand 1 (Cxcl1) measured in two different time-points, 30h and 48h after the injection (Ref. II, Figure 5B and D). At earlier time-point, 30 hours after subcutaneous injection and 6 hours of PMA treatment, miR-146a direct targets from the NF-κB pathway, Card10 and Irak1 also had reduced expression (Ref. II, Figure 5A). In addition, we detected reduced expression of chemokine Ccl11 (Ref. II, Figure 5D), which is known to attract eosinophils, neutrophils and macrophages and other inflammatory cells to the site of inflammation (Menzies-Gow et al. 2002, Su et al. 2005) and thereby to contribute to ICD-associated inflammation (Nakashima et al. 2014). Along with the suppression of ear-swelling (Ref. II, Figure 4C) and inflammation, the pre-injection of PF6-miR-146a nanocomplexes suppressed the expression of inter-cellular adhesion molecule 1 (Icam1) and also slightly the expression of tight junction protein Claudin 1 (Cldn1) mRNA (Ref. II, Figure 5C). Both, Icam1 and Cldn1 have previously found to be upregulated in damaged tissue in the case of

inflammation (Hua 2013). Consequently, also the improved integrity of epidermis was observed in mice injected with PF6-miR-146a nanocomplexes as compared to control injected mice (Ref. II, Figure 4E).

Interestingly, simultaneously to our study, subcutaneous injection of miR-146a mimic packed in transfecting agent Max Suppressor *in vivo* RNA-LANCEr II was demonstrated to alleviate psoriasiform skin inflammation in imiquimod-induced mice model of psoriasis further confirming that application of miR-146a *in vivo* into the skin indeed has anti-inflammatory effect (Srivastava et al. 2017). Another example of the effect of miRNA mimics *in vivo* in the skin is miR-203 which, in complex with *in vivo*-jetPEI, reduced tumor growth in a basal cell carcinoma (BCC) mouse model (Sonkoly et al. 2012). However, none of the studies demonstrated applicability of topical delivery of miRNA mimics or inhibitors and the question remains, whether this would also have an effect. Although in case of inflammation, the *stratum corneum* is damaged and tight junction between cells are open, allowing nanocomplexes or modified oligos more easily to penetrate, extensive amount of further research will be needed to develop topically applicable miRNA nanoformulations.

Still, several miRNA-based drug candidates for other cell types have been shown to be effective and have reached clinical trials. Accordingly, the wide-spread involvement of miR-155 in human cancers has led to many pre-clinical studies and clinical trials aiming inhibition of miR-155. For example, systemi-cally delivered anti-miR-155 polylysine-conjugated peptide nucleic acid (PNA) encapsylated in poly (lactic-co-glycolic-acid) (PLGA) polymer nanoparticles has been shown to result in reduced tumor growth in a mouse model of lymphoma (Babar et al. 2012). Moreover, synthetic miR-155 inhibitor, MRG-106 (miRagen Therapeutics, Inc.) has reached clinical trials in patients with CTCL (Foss et al. 2017, Bayraktar and Van Roosbroeck 2018). In addition to miR-155, miR-122 based drug miravirsen that inhibits HCV infection and targets chronic hepatitis C has entered into phase II clinical trial (van der Ree et al. 2014, Rupaimoole and Slack 2017).

In summary, we show in this study that PF6 formed homogenous nano-complexes with miR-146a mimics that efficiently entered into human primary keratinocytes, where miR-146a mimic inhibited the expression of its targets genes from NF-κB pathway and genes known to be activated by NF-κB. In addition, we show that PF6 can be used as a delivery vehicle for miRNAs *in vivo* in inflamed skin as subcutaneous pre-injection of PF6-miR-146a nanocomplexes suppressed the ear-swelling and reduced the expression of pro-inflammatory cytokines in a mouse model of ICD. We propose that CPP-based local delivery of therapeutic miRNA mimics has potential in treatment of inflammatory skin diseases.

4.3 PF14 efficiently delivers miRNA mimics into cancer cells (Ref. III)

It has been suggested that some miRNAs have anti-cancerous properties and their overexpression can be used to suppress tumorigenesis (Ji et al. 2009, Wiggins et al. 2010). By the time this study was initiated, miR-34a had already been described as a tumor suppressor miRNA with high therapeutic potential. Concordantly, many studies had shown miR-34a capacity to inhibit the expression of genes implicated in the control of cell proliferation (cyclins, CDKs), apoptosis (*BCL2, SIRT1*), senescence (E2F transcription factor 3 (*E2F3*)) or immune evasion (*PD-L1*) (Misso et al. 2014, Slabáková et al. 2017). In line with this, downregulation of miR-34a expression had been reported in multiple types of cancers, including leukemia (Chim et al. 2010), hepatocellular carcinoma (Li et al. 2009), pancreatic (Ji et al. 2009), prostate (Hagman et al. 2010) and lung cancer (Wiggins et al. 2010). On the other hand, restoration of its levels led to tumor suppressive effect by inhibiting cancer cell growth (Wiggins et al. 2010, Li et al. 2013).

Thus far, several therapeutic formulations of mostly lipid-based delivery vehicles had been shown to achieve efficient miR-34a delivery into various solid tumor cell types (Wiggins et al. 2010, Chakraborty et al. 2014). Regarding the development of cancer therapeutics, a compound MRX34 is well known as a miR-34a mimic encapsulated in a lipid carrier (Bader 2012). Although the mice studies showed significant tumor regression when treated with MRX34 nano-particles (Cortez et al. 2016, Daige et al. 2016), major drawbacks were encountered in a clinical study, where phase I trial was terminated due to the immune-related adverse events involving patient deaths, most probably caused by the delivery compound (Rupaimoole and Slack 2017). The key to develop efficient RNA-based therapeutics in cancer cells is to find alternative approaches for miRNA delivery to overcome toxicity associated with lipid-based formu-lations (Wittrup and Lieberman 2015).

In our study, we examined whether PepFects (PF14 in particular) could be used as potential vehicles for transporting miRNA mimics into the cancer cells. Our first objective was to test whether PF14 could deliver miRNA mimics (Dy547-labelled control miRNA, miR-34a-5p and control miRNA) into PPC-1 cell-line. Lipid-based transfection reagent siPORT, primarily designed for siRNA transfections, was used as a positive control. Analysis of fluorescent microscopy images showed that Dy547-labelled miRNA mimics were present in PPC-1 cells transfected either with PF14 or siPORT 24 hours after the transfection (Ref. III, Figure 1A). In addition, RT-qPCR analysis confirmed the presence of very high levels of miR-34a-5p in PPC-1 cells transfected with PF14-miR-34a-5p or siPORT-miR-34a-5p nanocomplexes as compared to control transfected cells (Ref III, Figure 1B).

As miRNAs target multiple pathways and regulate entire signaling networks by fine-tuning the expression of multiple genes, it is difficult in some cases to define the set of genes that is the most suitable for testing the effects of particular miRNAs.

Thus, we next performed transcriptome profiling to assess the effect of miR-34a in PPC-1 cells and to delineate the most suitable set of target genes to test miR-34a effect in further studies. In PPC-1 cells transfected with PF14-miR-34a-5p nanocomplexes, more than 3000 significantly affected genes were detected, while cells transfected with siPORT-miR-34a nanocomplexes, the number of influenced genes was less than 200 (Ref. III, Figure 2). We identified a strong downregulation of the nucleoporin 210 (*NUP210)* gene, among others, with both of the transfection reagents. Interestingly, NUP210 is identified as one of the most consistently upregulated proteins in numerous tumors (de las Heras et al. 2013). Another strongly downregulated gene was FOSL1 pseudogene 1 (*FOSL1P1*), a pseudogene of *FOSL1*. In addition to its pseudogene, FOSL1 itself was also downregulated more than 2-fold. Transcription factor FOSL1 has been identified as a direct and functional target of miR-34a in tumors (Wu et al. 2012, Yang et al. 2013).

Next, to validate the array results, we selected 5 downregulated genes (Rho GTPase Activating Protein 1 (*ARHGAP1)*, *AXL*, *CDC25a*, *FOSL1* and platelet derived growth factor receptor alfa (*PDGFRA)*) for RT-qPCR analysis. All of them belonged to the list of previously validated or predicted miR-34a targets (Misso et al. 2014, Chou et al. 2016). The downregulation of these 5 genes was verified independently by RT-qPCR in transfected PPC-1 and A549 cells (Ref. III, Figure 4). We suggest that these genes can be used as a set of markers to estimate the transfection efficiency of miR-34a.

Previously, PepFects have been shown to be highly efficient in NA delivery with minimal evidence of toxicity and immunogenicity (Suhorutsenko et al. 2011, Arukuusk et al. 2015). Therefore, we next assessed the efficiency and toxicity of PF14 as a delivery vehicle for miRNA mimic relative to three different lipid based transfection reagents (siPORT, LF2000 and RNAiMAX). Significant suppression of all five selected genes (*ARHGAP1*, *AXL, CDC25A*, *FOSL1*, *PDGFRA*) was observed in cells transfected with miR-34a-5p as compared to the control mimic with all four transfection methods (Ref. III, Figure 5A). Transfection with lipid-based reagent siPORT resulted in the least efficient suppression of gene expression, especially at higher cell density, while PF14 had similar or slightly lower transfection efficiency than LF2000 or RNAiMAX (Ref. III, Figure 5A). However, a significant decrease in cell viability was detected when control miRNA mimic was transfected with RNAiMAX or LF2000 (Ref. III, Figure 5B), whereas the viability of cells transfected with PF14-control miRNA were comparable with non-transfected control cells. Thus, although LF2000 and RNAiMAX result in efficient miRNA transfection, they also negatively affect cell viability.

In summary, we demonstrate that PF14 can be used as an efficient delivery vehicle for transporting miRNA mimics into cancer cells. In addition, by analyzing the transcriptome of PF14-miR34a transfected cancer cells, we delineate a set of genes, which is suitable for testing the effects of miR-34a mimic. Furthermore, we demonstrate that PF14 has similar transfection efficacy compared with lipofection methods and it has low impact on cell viability. Still, further studies are needed to test the PF14 capability to deliver miRNA mimics *in vivo* in the context of cancer therapy.

4.4 Modulation of complex interactions in tissue inflammation and cancer – lessons from the current study and future perspectives

The skin functions as both a physical barrier and an immunological organ that has evolved to defend living organisms against pathogens and physical dangers. Similarly, the airway epithelium, which is constantly exposed to a variety of environmental substances and pathogens, forms a critical barrier to the external environment. When risk factors emerge, the body triggers a complex inflammatory response through both epithelial cells and specialized immune cells (Dainichi et al. 2018). At the cellular level, the basis of inflammation is the activation of cascade-like gene expression networks that include hundreds of genes. Usually, cellular and molecular events efficiently minimize impending injury or infection during acute inflammatory responses and later the inflammation is resolved and tissue homeostasis is restored. However, uncontrolled acute inflammation may become persistent, contributing to a variety of chronic inflammatory diseases and also to cancer (Chen et al. 2017). It is estimated that one in five human cancers is related to chronic, unresolved inflammation caused by bacterial or viral infections or exposure to irritants. The other way around, oncogenes and tumor suppressors are also regulators of inflammatory pathways and their activation or loss in pre-malignant cells drives remodeling of inflammatory and tumor microenvironments through production of inflammatory mediators and recruitment of immune cells (Pesic and Greten 2016).

Three main pathways, NF-κB, MAPK and JAK-STAT, play major roles in inflammation and dysregulation of one or more of these pathways may lead to chronic inflammation and a microenvironment more prone to tumorigenesis (Chen et al. 2017). In the current study, we demonstrate the capacity of BNC2 to induce IFN-regulated genes with anti-cancerous characteristics, suggesting that reduced expression of BNC2 may lead to increased survival of cancer cells and upregulation of BNC2 may have tumor suppressor characteristics. However, as BNC2 increases IFN response, it is also possible that BNC2 may have limiting effect on viral infections, which has not been studied thus far. It has long been known that interferon-mediated antiviral responses through JAK/STAT signal transduction pathway are central to host defense against viral infection and induction of transcription of hundreds of IFN-stimulated genes further activate the immune response against viral infections. One of the key antiviral factors induced by IFN-signaling and found to be induced by BNC2 in this study, is 2',5'-oligoadenylate synthetase (OAS) family of genes (Choi et al. 2015). The OAS family is known to positively regulate the apoptosis, one of the ways how organism reacts to eliminate virus-infected cells. In our study we identified that all four members of OAS gene family were induced by BNC2, which could mean that BNC2 may contribute to antiviral defense through regulation of the expression of OAS family genes and apoptosis. BNC2 has been shown to be one of the genes activated by NF-κB (Yang et al. 2016), the latter playing a significant

role in the signal transduction that senses viral nucleic acids during pathogenic infection. That places BNC2 in an interesting crossroad position, where the possible regulation of anti-viral defense by BNC2 could be directed either through JAK/STAT and/or NF-κB signaling pathway. However, all these possible roles of BNC2 in viral response remain to be studied.

Another level of gene regulation associated with the current study is the effect of miRNAs. Here we used miR-146a as a model miRNA to target skin inflammation. Already initial studies demonstrated that miR-146a targets the NF-κB pathway (Taganov et al. 2006) and therefore, it is mainly known for its anti-inflammatory function. Interestingly, in association with the suppression of inflammatory responses, miR-146a functions in keeping control over the development of unwanted cancerous processes (Boldin et al. 2011). This has been shown in mice, where deficiency of miR-146a leads to development of auto-immune disorders and multiorgan inflammation and to gradual accumulation of hyperproliferating myeloid cells in secondary lymphoid organs driving myeloproliferative disease to progress to malignant myeloid sarcoma (Boldin et al. 2011, Zhao et al. 2011). In addition, an anti-tumor effect of miR-146a has been shown *in vitro* and *in vivo* in relation with myeloid leukemia, where injection of synthetic miR-146a mimic inhibited tumorigenic NF-κB activity and extended survival of acute myeloid leukemia (AML) bearing mice (Su et al. 2020). These studies confirm the presence of connections and interplay between chronic inflammation and cancerous processes.

Another miRNA that we were using to establish cell culture transfection model, miR-34a, is one of the most studied tumor suppressor miRNAs, frequently downregulated or absent in cancer and considered to have a high therapeutic value because it targets genes that are implicated in apoptosis and cell cycle regulation (Zhang et al. 2019). Although, these processes are not directly involved in the activation of inflammatory responses, they are still needed during resolution of inflammation, tissue regeneration and re-establishment of tissue homeostasis. Accordingly, a recent study has shown that miR-34a-deficient mice have impaired wound healing with delayed re-epithelization and augmented inflammation (Zhao et al. 2020).

In the second part of the study, we tested the therapeutic potential of anti-inflammatory miR-146a in skin inflammation and tumor suppressor miR-34a in cancer cells applying cell penetrating peptides. One obstacle in the use of nucleic acids as therapeutic molecules is their low penetration through the cell membrane (Arukuusk et al. 2013). CPPs may lead to a major breakthrough for the transport of nucleic acids, as they have been shown to successfully cross the cell membrane and deliver siRNAs and pDNA into different cell types (Veiman et al. 2013, Tai and Gao 2017). Compared to other translocation techniques, CPPs are able to enter cells in a noninvasive manner – they do not destroy the integrity of cellular membranes and are considered highly efficient and safe. However, their success as delivery systems *in vivo* has been limited. Our goal was to determine whether a new class of CPPs, called PepFects (PepFect6 and PepFect14) is suitable for delivering miRNAs into cells *in vitro* as well as *in vivo*. Until our study, PepFects

were proven to be efficient delivery vehicles for siRNA or pDNA transportation (Ezzat et al. 2012, Veiman et al. 2013), however, their utility in transporting miRNA mimics into cells and tissues had not been demonstrated before. Finding a suitable vehicle for miRNA delivery, which would be as efficient *in vivo* as *in vitro*, could benefit to develop miRNA-based therapeutic applications. Although our results show that PepFect-type CPPs are efficient in delivering miRNA mimics into primary keratinocytes and cancer cells and into inflamed skin *in vivo* in mice, it is still not clear whether miR-34a or miR-146a levels can be augmented enough to achieve therapeutic effect either in cancer treatment and/or to prevent uncontrolled inflammatory responses in diseased conditions. Also, we still do not know whether the delivery or the biological effect itself is limiting.

In conclusion, to develop successful therapeutic applications against chronic inflammatory diseases and/or cancer, the key tasks are to find either common or individual therapeutic targets or biological molecules that could be possibly used in the treatment. The second task is to develop efficient and safe delivery methods. It should be also noted that common denominators for the two disease processes may be very efficient as failure of resolution of inflammation initiates and promotes tumorigenesis and tumor expansion is closely connected to consequent inflammation.

5 CONCLUSIONS

I

The expression of a putative tumor suppressor gene, human Basonuclin 2 (BNC2), was demonstrated to be reduced in lung adenocarcinoma cell line A549 and in human squamous cell carcinoma patient samples. When overexpressed, BNC2 inhibited the proliferation of A549 cells and upregulated IFN-regulated genes with anti-cancerous characteristics. Overall, our data suggest that reduced expression of human BNC2 may lead to increased survival of cancer cells.

II

Cell-penetrating peptide PF6 was shown to form spherical and uniform nanocomplexes, ranging from 30–55 nm in diameter, with miRNA mimics, whereas miRNA complexes formed with other tested CPPs, PF14 and CADY, showed less regular morphology. With all three peptides, the formed miRNA nanocomplexes entered human primary keratinocytes, however, miR-146a down-regulated its target gene expression only when PepFects were used for the delivery. Moreover, subcutaneous pre-injection of PF6-miR-146a nanocomplexes reduced ear-swelling and the expression of pro-inflammatory mediators, IL-6 and Cxcl1 and cell-adhesion molecule Icam1 in mouse model of ICD. Our data together suggest that CPP-miR-146a nanocomplexes have potential in the suppression of skin inflammation *in vivo*.

III

PepFect14 was shown to transport miRNA mimics efficiently into cancer cells. More specifically, miR-34a-5p-delivered by PF14 was functional and down-regulated its target genes in PPC1 and A549 cell lines. A set of five genes, *ARHGAP1*, *AXL*, *CDC25A*, *FOSL1* and *PDGFRA*, was confirmed to be a reliable marker for estimation of miR-34a-5p transfection efficiency in cancer cell lines. In addition, in comparison with commercial transfection reagents, PF14 showed similar transfection efficacy, but it significantly less affected the cell viability.

SUMMARY IN ESTONIAN

Võimalikud terapeutilised lähenemisviisid põletikuliste protsessidega seotud signaaliradade moduleerimiseks

Kõik elusorganismid, alates lihtsatest eeltuumsetest kuni keerukate mitmerakuliste eukarüootideni, on pidevas kokkupuutes väliste ja sisemiste ohuteguritega. Epiteelkoed, mis katavad enamuse hulkraksete välispinnast ja piiritlevad ka selle organeid, on ühtlasi esimeseks kaitseliiniks keskkonnategurite ja paljude patogeenide vastu. Ohutegurite ilmnemisel käivitab organism nii epiteelrakkude kui ka spetsialiseeritud immuunrakkude vahendusel komplekse vastuse põletikureaktsioonina, mille eesmärk on tuvastatud ohutegurite kõrvaldamine, immuunsüsteemi aktiveerimine ning kudede ja organismi kui terviku homoöstaasi taastamine. Rakutasandil hõlmab põletik keerukate süsteemide rakendamist ja koordineerimist, mille käigus aktiveeritakse sadu geene minutite jooksul peale esimest kokkupuudet ohuteguritega. Kui aga esmane põletikureaktsioon osutub ebaoptimaalseks ja kaasatakse ka omandatud immuunsüsteemi aktiveerimine, võib see muutuda kas ülemääraselt ägedaks või krooniliseks ning viia kudede ulatuslikule kahjustusele. Püsiv kokkupuude teatud välisteguritega suurendab kroonilise põletiku riski ning vastavad haigused võivad aja jooksul kujuneda kasvajalise protsessi eelfaasiks.

Käesoleva doktoritöö peamine eesmärk oli täiendada alusteadmisi epiteliaalsete kudede kaitsemehhanismidest ja uurida, kas kullerpeptiidid võiksid olla sobivad kandurid mikroRNA miimide viimiseks rakkudesse ja põletikulistesse kudedesse.

Töö esimeses osas seati eesmärgiks iseloomustada potentsiaalse kasvaja supressorgeeni, inimese basonukliin 2 (*BNC2*) rolli kopsuvähi patogeneesis. Inimese kromosomaalse piirkonna 9p22 kaardistamise käigus tuvastasime tsinksõrme motiive sisaldava uue geeni, *BNC2*. Mitmetes töödes on näidatud, et *BNC2* ekspresseerub laidaldaselt paljudes erinevates kudedes ja epiteliaalse päritoluga rakuliinides, kuid on maha surutud mitmetes vähivormides. Sellest tulenevalt otsustasime uurida *BNC2* ekspressiooni epiteliaalse päritoluga kopsuvähi koeproovides ja rakuliinis. Tulemustest selgus, et inimese *BNC2* ekspressioon on vähenenud nii kopsu adenokartsinoomi rakuliinis A549 kui ka lamerakulise kartsinoomi diagnoosiga patsientide koeproovides. Reeglina on tuumorsupressorgeenid vähis allareguleeritud, seega on taolise geeni ekspressiooni taastamine üks potentsiaalne võimalus vähi ravis. Meie katsed näitasid, et BNC2 üleekspressioon A549 rakuliinis pärssis vähirakkude vohamist.

Tsinksõrme motiividega transkriptsioonifaktorid reguleerivad sageli sadade geenide ekspressiooni, seondudes DNAle ning seeläbi suunates raku üldisi füsioloogilisi protsesse nagu raku jagunemine, diferentseerumine ja apoptoos. BNC2 kui transkriptsioonifaktori mõju iseloomustamiseks hindasime *BNC2* üleekspressiooni toimet raku transkriptoomile, kasutades mikrokiibi analüüsi ja signaaliradade otsingut. Leidsime, et BNC2 mõjutab oluliselt eelkõige interferoonide poolt aktiveeritavaid signaaliradasid, sealhulgas ka geene, mis teadaolevalt reguleerivad kasvajarakkude paljunemist ja apoptoosi. Seega võib öelda,

et inimese *BNC2* geen käitub epiteelirakkudes tõenäolise kasvaja supressor-geenina ning tema madalam ekspressioonitase võib aidata kaasa inimese epiteliaalsetest kudedest lähtunud kopsuvähi tekkele.

Doktoritöösse kaasatud järgnevate artiklite eesmärk oli hinnata mikroRNAde (miRNA) võimalikku rakendatavust ravimiarenduses, mis on suunatud kasvaja-liste protsesside või krooniliste põletike pidurdamisele epiteelkoes, sealjuures kasutades varem teadaolevaid siRNA transpordiks sobilikke kullerpeptiidide.

MikroRNAd on väikesed valku mittekodeerivad RNA molekulid, mis seostuvad märklaud-mRNAga ning vaigistavad viimase ekspressiooni. Tänu oma-dusele reguleerida korraga paljude geenide ekspressiooni mängivad miRNA-d olulist rolli paljudes bioloogilistes protsessides, sealhulgas põletiku kujunemisel ning vähi arengus. Sellest tulenevalt arvatakse, et miRNAde inhibeerimine või manustamine võib omada terapeutilist potentsiaali. Üheks suurimaks takistuseks miRNAde kui terapeutiliste molekulide kasutamisel on olnud nende ebaefek-tiivne transport haigusest kahjustatud kudedesse või rakkudesse. Meie tööhüpo-teesiks oli, et miRNAde transportimiseks rakkudesse on võimalik kasutada kullerpeptiide, mis on võimelised läbima rakumembraane ja viima miRNA sihtmärkgeenideni. Eelnevates töödes on edukalt näidatud, et kullerpeptiide on võimalik kasutada erinevate nukleiinhapete, sh siRNAde ja plasmiidse DNA transpordiks rakku *in vitro* ja *in vivo*, kuid nende kasutatavust miRNA miimide transpordiks rakkudesse ei olnud varem näidatud.

Käesoleva doktoritöö teises osas näitasimegi, et valitud kullerpeptiidid suuda-vad moodustada mittekovalentseid komplekse ka miRNA miimidega ning transportida need inimese primaarsetesse naha keratinotsüütidesse *in vitro* ja hiire naharakkudesse *in vivo*. Kõik kolm valitud peptiidi (PF6, PF14 ja CADY) pakkisid miRNA miimi stabiilseteks nanopartikliteks ning transportisid selle rakkudesse, kuid vaid kahe esimese puhul neist toimus miRNA vabanemine kompleksist ja märklaudgeenide vaigistamine. Lisaks näitasime hiire ärritus-kontaktdermatiidi mudelis, et PF6-mikroRNA-146a nanokomplekside süstimine naha alla alandab põletikulist turset ning vähendab põletikumediaatorite ekspres-siooni ning et miR-146a miimide lokaalne manustamine on põletikuvastase toimega. Kokkuvõttes võib öelda, et PF-tüüpi kullerpeptiidid võivad olla aluseks miRNA-põhiste ravimeetodite väljatöötamisele.

Töö kolmanda osa eesmärk oli hinnata PF14 võimet transportida vähi-rakkudesse vähivastaste omadustega miRNA-34a-5p miime ning leida geenide komplekt, mis sobiks miRNA-34a-5p transfektsiooni efektiivsuse hindamiseks. Meie katsed näitavad, et PF14 suudab transportida miRNA-34a-5p miime ees-näärme vähi (PPC-1) ja kopsu adenokartsinoomi (A549) rakuliinidesse. Reaalaja kvantitatiivne PCR pärast kullerpeptiidiga transporti tõendas miRNA-34a-5p ülikõrget taset neis rakkudes. Analüüsides PF14-miR-34a-5p-ga transfekteeritud rakkude transkriptoomi, tuvastasime ka geenid, mida saab kasutada transfekt-siooni efektiivsuse hindamiseks. Seega leidsime, et PF14 on efektiivne kandur miRNA miimide transpordiks vähirakkudesse. Lisaks lubavad meie tulemused väita, et miRNA miimide puhul on kullerpeptiidid efektiivsuselt võrreldavad levinud liposoomipõhiste transfektsioonimeetoditega, kuid neil on vähem kõrval-toimeid.

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