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ABSTRACT

Endometriosis is a frequent gynecological disease that influences up to 10% of women in reproductive age. The disorder is defined by the presence of endometrial implants outside the uterine cavity that cause chronic pelvic pain and often infertility. The current diagnostic and therapeutic strategies for endometriosis are limited. The treatment often lacks long-term efficacy and causes adverse effects due to systemic administration of pharmaceutics. Therefore, novel treatment strategies for endometriosis are needed. Many highly potential therapeutic targets are in fact located inside the diseased cells. Thus, the biggest challenge for successful treatment of various conditions is that the therapeutic candidates have to reach their intracellular target molecules at the intended target sites. Hence, the objective of this study was to develop or repurpose peptide-based drug carriers for nanomedical applications mainly focusing on endometriosis.

To start with, highly potent drug delivery vehicles, cell-penetrating peptides (CPPs) were used as oligonucleotide carriers that aim to adjust the therapeutic gene expression levels. First, a series of histidine-containing peptides was designed that display adaptive response to the physical environment. The most successful peptide, pH-sensitive NickFect70 was highly efficient in transporting short interfering RNA (siRNA) into cells both in vitro and in vivo. Second, to test the therapeutic potential of CPP/siRNA nanoparticles, the efficacy of siRNAs against two genes related to the growth and progression of diseased tissues was investigated in cancer and endometriosis monolayer and 3D spheroid cell cultures. This resulted in the reduction of cellular proliferation, invasion, and migration. In addition, combining gene therapy and hormonal treatment approaches proved to mediate synergistic suppression of proliferation and invasion of endometriotic cells. Despite being very efficient in cellular internalization, most CPPs are not tissue specific and might lead to unspecific accumulation of therapeutics in healthy organs when administered in vivo. Since the aim of this work was to develop proof-of-concept nanomedicine applications for endometriosis, as a final, third, step a series of specific tissue targeting peptides was investigated. Targeting specific isoforms of angiogenic extracellular matrix proteins fibronectin and tenascin overexpressed in solid tumors with PL1 homing peptide proved to be most successful candidate for endometriosis targeting as well. Silver nanoparticles functionalized with synthetic PL1 peptide showed specific accumulation to 2D and 3D cell cultures and clinical endometriosis tissue samples.

In conclusion, the results of this thesis proved the relevance of the knockdown of two therapeutic genes by CPP/siRNA nanoparticles for endometriosis treatment. Therapeutic effects were enhanced by the combination with a hormonal drug. In addition, PL1-guided nanoparticles showed high potential in precision diagnosis and tissue specific therapy of endometriosis. All in all, the findings of this thesis propose several peptide-based nanomedical applications for the treatment of endometriosis that suggest translational relevance.

TABLE OF CONTENTS

LIST OF PUBLICATIONS

The following publications form the basis of this dissertation and are referred to in the text using Roman numerals:

I Porosk L, Arukuusk P, Põhako K, Kurrikoff K, **Kiisholts K**, Padari K, Pooga M, Langel Ü (2019) Enhancement of siRNA transfection by the optimization of fatty acid length and histidine content in the CPP. Biomater Sci 7:4363–4374

II **Kiisholts K**, Kurrikoff K, Arukuusk P, Porosk L, Peters M, Salumets A, Langel Ü (2021) Cell-Penetrating Peptide and siRNA-Mediated Therapeutic Effects on Endometriosis and Cancer In Vitro Models. Pharmaceutics 13:1618

III Simón-Gracia L*****, **Kiisholts K***, Petrikaitė V, Tobi A, Saare M, Lingasamy P, Peters M, Salumets A, Teesalu T (2021) Homing Peptide-Based Targeting of Tenascin-C and Fibronectin in Endometriosis. Nanomaterials 11:3257

***** Shared first authors

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The author's contribution to each article is as follows:

I participated in in vivo experiments, tissue processing and qRT-PCR data collection and analysis;

II designed most of the experiments, participated in performing the 3D spheroid tissue penetration experiments, performed the gene expression analysis, immunocytochemistry, cell cycle analysis, invasion assessment, nanoparticle-danazol co-treatment, 3D spheroid migration assay, analyzed most of the data and wrote the manuscript.

III participated in the design of the experiments, performed the phage binding, immunohistochemistry and tissue binding experiments, participated in performing the immunocytochemistry and flow cytometry experiments and participated in both the data analysis and in the writing of the manuscript;

Other publications:

IV Veiman K-L, Künnapuu K, Lehto T, **Kiisholts K**, Pärn K, Langel Ü, Kurrikoff K (2015) PEG shielded MMP sensitive CPPs for efficient and tumor specific gene delivery in vivo. J Controlled Release 209:238–247

V Künnapuu K, Veiman K, Porosk L, Rammul E, **Kiisholts K**, Langel Ü, Kurrikoff K (2018) Tumor gene therapy by systemic delivery of plasmid DNA with cell-penetrating peptides. FASEB BioAdvances 1:105–114

ABBREVIATIONS

3D	three-dimensional
AgNP	silver nanoparticle
AON	antisense oligonucleotides
CendR	C-end rule
CPP	cell-penetrating peptide
DAXI	DaxibotulinumtoxinA for Injection
DLS	dynamic light scattering
ECM	extracellular matrix
EMA	European Medicines Agency
EMS	endometriosis
EPR	enhanced permeability and retention effect
FACS	fluorescence-activated cell sorter
FDA	Food and Drug Administration
Fmoc	fluorenylmethyloxycarbonyl
Fn-EDB	fibronectin Extra Domain-B
GnRH	gonadotropin-releasing hormone
GFP	green fluorescent protein
HDA	heparin displacement assay
ICC	immunocytochemistry
IF	immunofluorescence
IHC	immunohistochemistry
iRGD	internalizing RGD
i.v.	intravenous
KD	dissociation constant
Luc	luciferase
mRNA	messenger RNA
miRNA	microRNA
MMAE	monomethyl auristatin E
MMP	matrix metalloproteinase
MR	molar ratio
MRI	magnetic resonance imaging
NRP-1	neuropilin-1
NF	NickFect
NGS	next-generation sequencing
NP	nanoparticle
ON	oligonucleotide

pDNA	plasmid DNA
PEG	polyethylene glycol
PEI	polyethyleneimine
PF	PepFect
PI	propidium iodide
pre-RISC	precursor RNA-induced silencing complex
qRT-PCR	quantitative real time PCR
RBC	red blood cells
RISC	RNA-induced silencing complex
RNAi	RNA interference
RNAiMAX	Lipofectamine™ RNAiMAX
RR	ribonucleotide reductase
RRM2	ribonucleotide reductase M2 subunit
RT	room temperature
siRNA	small interfering RNA
Tat	transactivator of transcription
TEM	transmission electron microscopy
TNC-C	tenascin C C-isoform
TP10	transportan10
TPP	tumor penetrating peptides
UT	untreated
VEGF	vascular endothelial growth factor

INTRODUCTION

Endometriosis is a hormone-dependent and neuroinflammatory gynecological disorder with the main symptom being chronic pelvic pain. The functional endometrial-like tissue grows distinctively outside the uterus, mainly on the abdominal-pelvic organs and lining of the cavity. Up to date, there is no effective cure for endometriosis and current pharmaceuticals only alleviate the symptoms, whereas the resection of the lesions often leads to recurrence. Although endometriosis is considered a benign condition, it exhibits similar behavior to cancer, e.g., increased cell invasion, migration, tissue inflammation and angiogenesis. Therefore, several potential targets for endometriosis were investigated here based on previous cancer treatment research.

Nanomedical approaches are powerful tools for the development of new treatment strategies for various conditions, since it can be directed towards specific tissues or many conventionally undruggable targets. For example, in case of gene therapy, therapeutic oligonucleotides can regulate the expression of countless genes in most types of cells. Nevertheless, most nucleic acids or macromolecular drugs are not able to independently reach their intracellular targets. Thus, for successful therapy, the penetration through the cell membrane remains the biggest challenge for the drug molecules. To aid that, it is essential to develop biocompatible and nontoxic delivery vehicles that are capable of encapsulating the therapeutic cargo, accumulating in the diseased tissue, and being effective in cellular delivery and endosomal drug release. Thus, the formulations of two different types of cargo delivery peptides and their proof-of-concept preclinical therapeutic applications were investigated in this thesis.

First, a new series of highly potential drug delivery vehicles, cell-penetrating peptides, aiming to improve siRNA transport into cells both in vitro and in vivo, was being developed. Thereafter the most successful peptides and their nanoparticles with therapeutic siRNA were tested in several preclinical disease models like cancer and endometriosis. The second type of peptides studied was the homing peptides, that are intended for systemic administration and developed to specifically target tissues of interest. Their use may help to overcome the limiting systemic side effects and unspecific accumulation of therapeutics in healthy organs. Therefore, as a final step a series of tissue targeting peptides was investigated to identify a homing peptide specifically targeting endometriosis. Monolayer and spheroid cell cultures and clinical tissue samples of endometriosis lesions were used for the testing of peptide-guided nanoparticles' uptake and proof-of concept therapeutic efficacy.

1. LITERATURE OVERVIEW

1.1 Endometriosis

Endometriosis is a hormone-dependent and neuroinflammatory chronic gynecological condition that is defined by the growth of functional endometrial-like tissue outside the uterine cavity [1,2]. Often there are multiple lesions distributed throughout the abdominal-pelvic peritoneum or visceral organs and that is the cause for the main symptom of endometriosis – chronic pelvic pain [3]. Endometriosis affects up to 10% of women in reproductive age [4,5], but surprisingly the overall awareness among public and health care practitioners is low. Furthermore, although endometriosis is more frequent than cancer among women in reproductive age, it has constantly received insufficient research funding [6]. Endometriosis is not considered a fatal condition, nevertheless, it has a major negative effect to the life quality of the patients. The symptoms can be very heterogeneous and distressing, including various types of pain: dysmenorrhea (during menstruation), dyspareunia (during sexual intercourse), dyschezia (during defecation), dysuria (during urination) and what is more, endometriosis is a frequent source of infertility [1,7–9].

1.1.1 Anatomic locations and histology of the lesions

Endometriosis lesions have been found from different locations in the peritoneal cavity (Figure 1). The most frequent locations for the lesions are the ovary, uterosacral ligament, Pouch of Douglas and the bladder [10]. When the endometrial cells have established outgrowths in the myometrial layer of the uterus, the pathology is called adenomyosis [11].

Based on the nature of the lesions, three main phenotypes of endometriosis can be defined: ovarian, peritoneal and deep infiltrating endometriosis. In case of ovarian endometriosis, the endometriomas, which are cysts surrounded by endometrial cells, may form located on the surface of the ovaries. Endometriomas contain dark brown old menstrual blood and are therefore often referred to as chocolate cysts. The other two types may involve any space or organ within the pelvis, but the distinction is that the peritoneal lesions are superficial and the lesions where endometriotic tissue has penetrated more than 5 mm in depth, is classified as deep infiltrating endometriosis. The most common type is considered to be the endometrioma, which is often accompanied by superficial peritoneal lesions [10].

In terms of histology and cellular composition, endometriotic lesions often have both endometrial components: epithelial glands and stromal cells surrounding the glands (Figure 2). In some cases though, it has been shown that only stromal cells are present in the lesion [12].

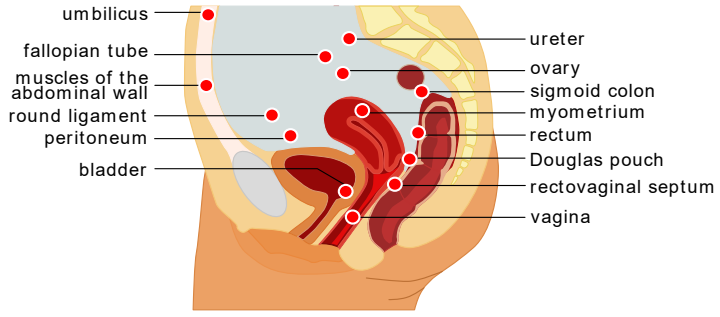

Figure 1. Frequent anatomic locations of endometriosis. Modified from Wikipedia [13].

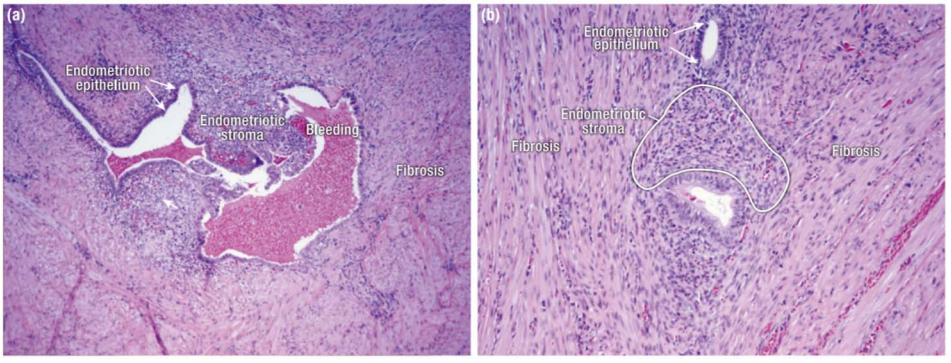

Figure 2. Hematoxylin and eosin staining of typical endometriosis lesions. Reprinted with permission from Bulun et al., Copyright 2019 Oxford University Press [14].

1.1.2 Pathogenesis

To date, the fundamental pathogenetic mechanisms of endometriosis have not been identified. There are several hypotheses and theories proposed, though. The most popular and widely accepted Sampson's theory suggests that the endometrial cells reach the peritoneal cavity during retrograde menstruation. More specifically, that a proportion of menstrual blood flows backwards through the fallopian tubes and ends up in the pelvis. Another hypothesis proposes the endometrial cells' dissemination similar to cancer metastasis, using the vascular and lymphatic system. The celomic metaplasia theory explains that the wall of the main body cavity (celomic wall) is embryonically connected to Müllerian ducts that later evolve into female reproductive system. Therefore, the endometriotic cells are alleged to be able to form lesions in all celomic wall derivative tissues. There is also the undifferentiated stem cell recruitment theory that has two different variations. The first claims that the stem cells in the bone marrow and second that the stem cells in the uterine endometrium are responsible for the endometriosis lesion formation. After the stem cell migration, different hormones and molecular factors in site of the forming lesion are said to be behind the differentiation of the

stem cells to endometrial cells. The embryogenetic theory claims that the residues of Wolffian or Müllerian duct tissues may dislocate during a critical period of uterine morphogenesis and that in later life these embryonic cells may contribute to the endometriotic lesion formation in response to estrogen exposure. All theories have some evidential clinical or experimental proof, but on the other hand some contradictions, therefore the relying mechanism behind the primary formation and development of the endometriosis lesions remains unclear for now [15].

1.1.3 Diagnosis and management

The current diagnosis and treatment strategies for different endometriosis phenotypes are very limited. For diagnosing ovarian endometriomas and deep infiltrating endometriosis, imaging techniques such as transvaginal ultrasound and magnetic resonance imaging (MRI) can help. The only option for the diagnosis of superficial peritoneal lesions is exploratory laparoscopic surgery that is followed by histologic confirmation of the disease. The lack of non-invasive biomarkers and the need for using expensive and invasive laparoscopy procedure restricts and delays the diagnosis. Starting from the presentation of the first symptoms, the duration of the diagnosis delay might be unreasonably long, up to 8–12 years [16].

There is no actual cure for endometriosis and the current treatment can only alleviate the symptoms of the disease [17]. The treatments include painkillers, laparoscopy to resect the endometriosis lesions and hormonal treatments such as contraceptives (progesterone and estrogen), progestins or gonadotropin-releasing hormone (GnRH) analogues [17,18]. On the whole, the pharmaceutical treatment often lacks long-term efficiency and causes adverse effects due to systemic administration [17]. Hormonal treatment has shown to be effective in controlling the chronic pelvic pain, but it is not applicable if the patient is seeking for pregnancy [18]. Although laparoscopy can temporally remove the lesions and relieve the symptoms, complete excision of the lesions might be difficult and there is a high percentage of lesion recurrence after some time [19]. One of the most extensively used hormonal treatments used to be a synthetic androgen danazol, that has been successful in pain relief and even lesion regression, but it has severe hyperandrogenic side effects. Therefore, prolonged danazol usage is not possible, the same goes for the ovarian suppressing GnRH analogues, that are nevertheless used as a second line treatment, when other remedies have failed [18].

All in all, there is a pressing need for the development of new non-invasive diagnostic and effective treatment strategies for endometriosis.

1.1.4 Functional properties and microenvironment

Endometriotic cells exhibit functional characteristics like adhesive, proliferative, migrative and invasive properties; they are responsive to hormonal stimuli and resistant to apoptosis [20,21]. The tissue microenvironment often instructs the fate and behavior of the cells and plays an important role in the disease onset and progression. The microenvironment consists of different components like neighboring cells, soluble chemical factors, the extracellular matrix (ECM) and biophysical fields (Figure 3) [22]. The ECM components of the endometriosis lesions have shown to differ from the surrounding tissue [23,24], which could influence the adhesion, proliferation and migration of the misplaced cells. The overall microenvironment of endometriosis includes the presence of various immune components and it is supporting inflammation, angio- and neurogenesis. It could also affect the growth and the differentiation ability of the endometriotic cells by changing the gene expression through epigenetic modulations like DNA methylation, histone modifications or microRNA-induced gene silencing.

At the molecular level, at least four altered pathways relevant to endometriosis and its attachment site cross-talk have been identified: cellular injury (ubiquitin/proteasome), inflammation (NFκB), tissue remodeling (TGF-β) and cellular proliferation (KRAS) [25]. There is also strong proteomic evidence showing extensive metabolic reprogramming of endometriotic cells, called the Warburg effect, where there is substantial upregulation of glycolysis and downregulation of oxidative respiration present. This is a widely known metabolic phenotype previously described to be acquired by many types of cancers [26].

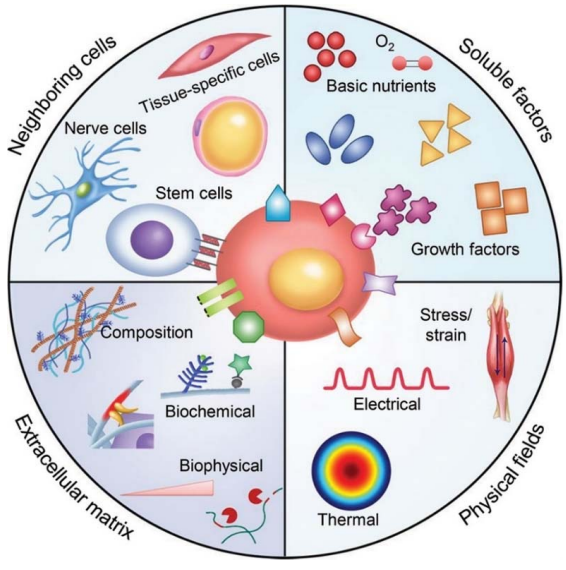

Figure 3. Schematic representation of the main components of the cell microenvironment. Reprinted with permission from Huang et al., Copyright 2017 American Chemical Society [22].

1.2 Cell culture models for primary in vitro testing of drugs

1.2.1 Monolayer cell cultures

There are multiple ways of establishing cell cultures. First, it is possible to directly extract cells from live normal tissue or pathology of interest and maintain them in a suitably supplemented cell culture media. The cultures created this way are called primary cell cultures. These cultures have the highest similarity to the actual tissue, but on the other hand, most primary cell types have several limitations like slow and limited proliferation rate.

When it is necessary to propagate cells for a longer period of time or use high number of cells and e.g., compare different drug responses and undertake different efficacy assays, it is necessary to use immortalized cell lines instead of primary cells. The first human cell line was developed in the year 1951 from cervical carcinoma tissue and was named HeLa, after the name of the cancer patient [27]. The number of established immortalized cell lines has massively grown and become commercially available since that.

The cell cultures provide easily accessible and alterable environment where monitoring the biological changes is relatively easy, since there is a large set of cell culture assays developed and available. That is the main reason, why in the process of drug development, one of the first biological feasibility steps frequently is cell culture testing.

1.2.2 3D cell cultures

Regular monolayer cell cultures are still artificial and lack several characteristics of the live tissue, where the cells exist within a dynamic three-dimensional (3D) microenvironment. There are cell-cell interactions through the entire cell surface, different intercellular signaling pathways, tissue structures etc. It is widely accepted that compared to culturing cells as monolayers on tissue culture plastics, 3D tissue culture mimics many aspects of the in vivo situation better, like gene expression patterns, protein synthesis, cell morphology and differentiation [28]. Furthermore, 3D cultures have shown to be more drug resistant and also, they enable the evaluation of a major challenge in drug development – the tissue penetration ability. It is common that many drugs showing good efficacy in regular cell culture tests fail to perform already in the phase of 3D experiments. Thus, the 3D cell cultures can be considered as an intermediate step between regular monolayer cells and in vivo experiments, which enables the reduction of experimental animals used in the drug development research [29]. Therefore, advanced, multilayered 3D cellular models have been used in this work for disease modelling.

1.3 Gene therapy

Gene therapy is a highly potential approach for treating a wide range of different diseases, genetic disorders and malignancies. During gene therapy, the target cells are being genetically modified or their gene expression is regulated in a desired direction by using therapeutic nucleic acids (Figure 4) [30]. The over-expressed genes in the diseased cells can be downregulated by post-transcriptional silencing using single-stranded antisense oligonucleotides (AON) or double-stranded microRNA (miRNA) and short interfering RNA (siRNA). It is also possible to induce specific gene expression or upregulate genes by the delivery of circular plasmid DNA (pDNA), linear messenger RNA (mRNA) or the CRISPR-Cas system. The latter also enables inducing permanent gene knockouts by inducing deletions in the genomic DNA or even gene repairing by precise sequence replacement [31]. Gene therapy offers many potential advantages over conventional small molecule-based systemic pharmacological treatments. First, it enables the regulation of specific target genes and can also provide a solution for influencing proteins that are considered undruggable by classic pharmacological treatment. What is more, the gene therapy tools are in a manner of speaking, programmable; their therapeutic target can be changed without changing the in vivo pharmacokinetics [32].

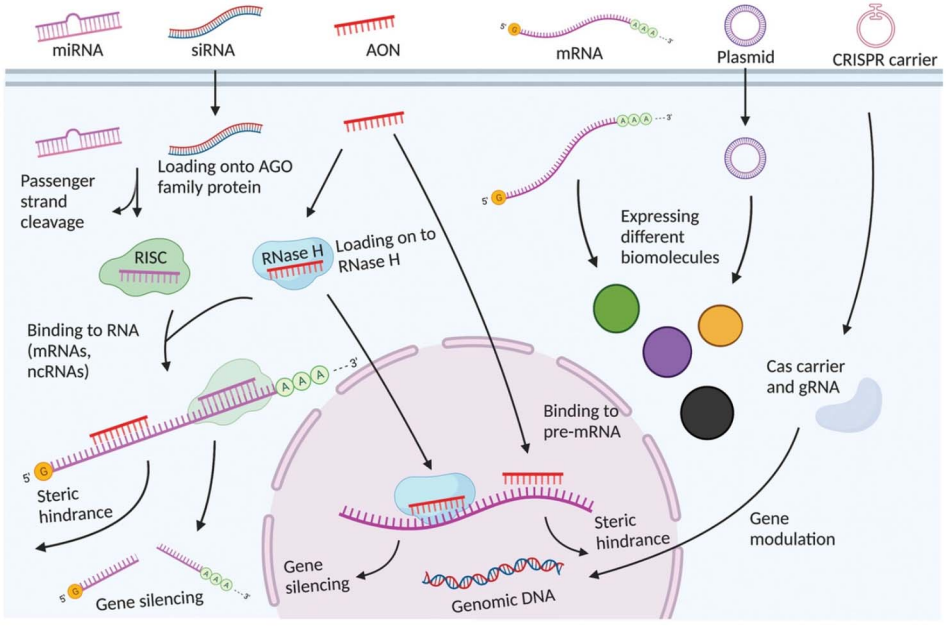

Figure 4. Schematic illustration of the main gene therapy tools. Reprinted from Luo et al. [30].

1.3.1 RNA interference

The discovery of the regulatory properties of small noncoding RNA molecules has significantly widened the overall understanding of the eukaryotic gene regulation and function. One of the most important pathways the regulatory RNA uses, is the RNA interference (RNAi) pathway, which leads to highly specific gene silencing triggered by the regulatory RNA molecules binding to complementary mRNA. Since its discovery in 1998 [33], RNAi has been regarded as one of the most promising gene therapy approach. The principal pathway of RNA interference is shown on Figure 5.

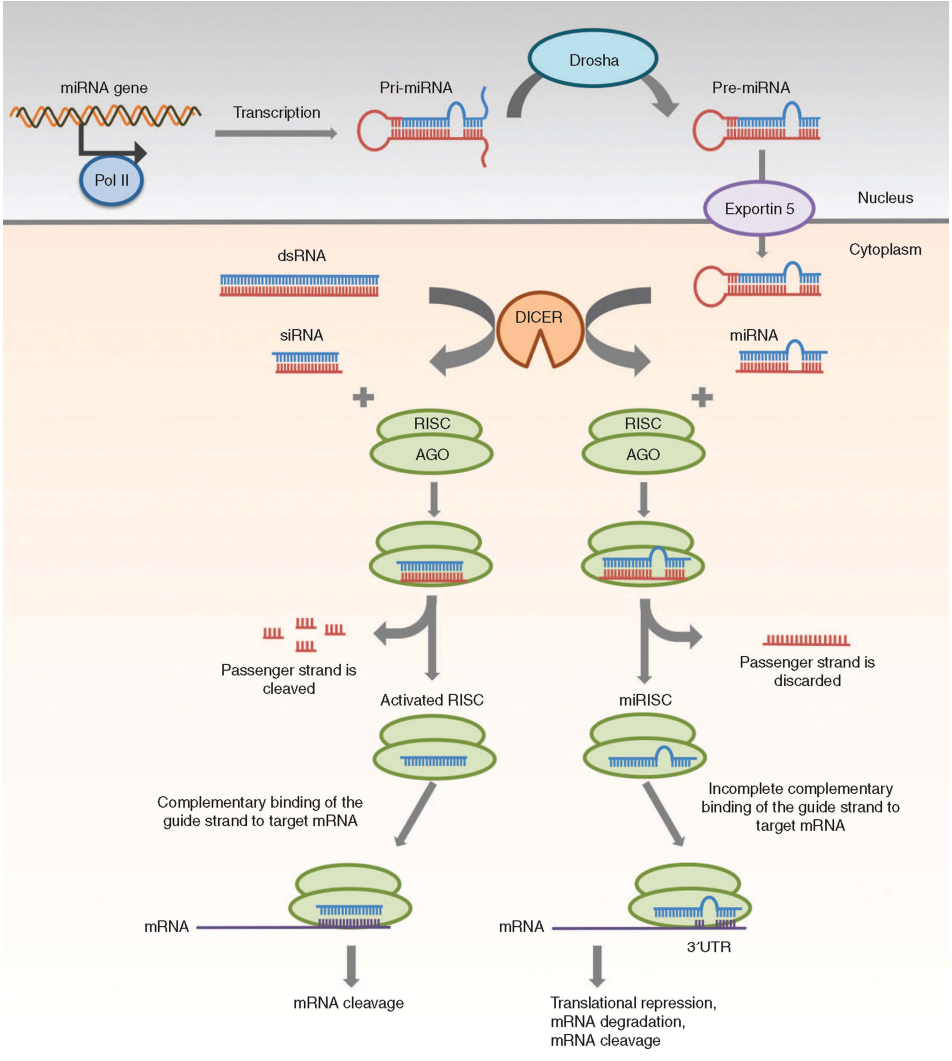

Figure 5. The RNA interference pathway: gene silencing mechanisms of siRNA and miRNA. Reprinted with permission from Lam et al., Copyright 2015 Official journal of the American Society of Gene & Cell Therapy Published by Elsevier Inc [34].

The RNA molecules behind mRNA silencing mechanism are partially complementary miRNA and siRNA that is usually fully complementary to the target mRNA. Once reached to the cytoplasm of the cells, miRNA or siRNA binds to the precursor RNA-induced silencing complex (pre-RISC). There, the passenger strand of the duplex is removed. The mature RISC complex is containing the guide strand that can then be bound to the target mRNA and induce the post-transcriptional gene silencing.

The most common strategy for RNAi induction is delivering 21–22 base pairs long siRNA molecules. There are various software tools available for rational siRNA design. To increase the RNA stability, it is possible to induce various modifications to the siRNA molecule. For example, introducing modified bases and sugars to the sequence, altering the molecule length and the nature of 5' and 3' ends. The most usual for the latter are the overhangs of deoxynucleotides at one or both ends. Another possibility, that has shown improved silencing at times, is introducing longer, 25–27 base pairs long double-stranded RNA to the cells. These would further be processed and cleaved by the intracellular Dicer enzyme [35].

1.3.2 siRNA target genes

1.3.2.1 VEGF

The invasiveness and angiogenic potential of the endometriotic implants are the most important factors for establishing a functional ectopic lesion of endometriosis. In new forming blood vessels, a process called neovascularization, the most important gene expressed is vascular endothelial growth factor (VEGF). Due to extensive heterogeneity of endometriosis in terms of the lesions' location, gene expression profiles and microenvironment, only a limited number of therapeutic targets have been proposed, while VEGF being one of the most frequent ones [14]. A variety of VEGF blockers and inhibitors have been investigated for therapeutic purposes in experimental endometriosis models in vitro and in vivo. Consequently, several in vivo studies have been successful in regression of the lesions by reducing their blood supply [36,37].

1.3.2.2 RRM2

Ribonucleotide reductase (RR) is expressed in all proliferative cells and it is a limiting enzyme for DNA synthesis and replication. For the enzyme to be active, both of its subunits, M1 and M2 have to be expressed [38]. Although endometriosis is a proliferative disease, so far, the importance of ribonucleotide reductase has not been investigated. For tumor progression though, the RR overexpression has been shown to be one of the key factors by also increasing cancer cells' angiogenic and invasive potential [39]. Several RR inhibitors are investigated in the clinical trials against tumor progression or even actively used in oncologic treatments, e.g., gemcitabine [40]. For these reasons, the potential of using ribonucleotide reductase M2 subunit (RRM2) as a therapeutic target for endometriosis was investigated in the current thesis.

1.4 Drug delivery methods and challenges

The biggest challenge for biologically active nucleic acids and macromolecular drugs is reaching their intracellular targets. These therapeutic molecules are commonly hydrophobic and/or negatively charged and therefore are not able to independently penetrate the cell membrane. Therefore, it is essential to find a suitable delivery vehicle that would be capable of encapsulating the drug or oligonucleotide (ON) into nanoparticles (NP), be effective in cellular delivery and at the same time non-toxic to the cells and tissues.

There is a large variety of organic drug delivery systems designed and available nowadays: viral vectors, peptides, antibodies, liposomes, dendrimers, polymers, extracellular vesicles etc. Each system has their own characteristic advantages and disadvantages. In this thesis, different peptide-based delivery vectors were used. Their advantages include high efficacy in drug and ON encapsulation and delivery, low toxicity and immunogenicity, high cost-effectiveness, relatively easy and quick synthesis.

On Figure 6, the extracellular and intracellular barriers limiting the drug delivery are shown, representing liposomal gene delivery as an example. Regardless of the delivery vehicle, the biological challenges for drug delivery in case of intravenous administration are mainly the same. The NPs have to survive the degradative enzymes in the blood, bypass the opsonization by serum proteins and escape the immune components. Thereafter the NPs need to cross different biological membranes by entering the cells, escaping the endosomes and reaching the nucleus, if needed [41].

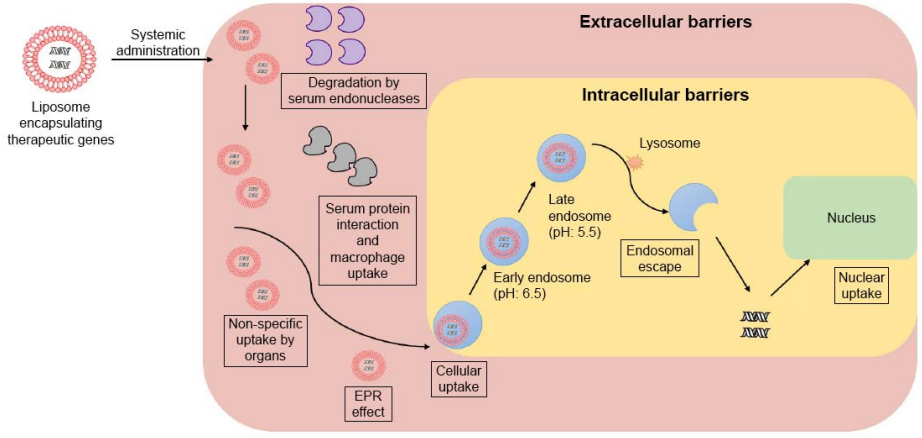

Figure 6. Representation of the extracellular and intracellular barriers limiting drug delivery. Modified from Altwaijry et al. [41].

There are two different strategies for in vivo drug delivery, namely passive and active. The first relies on passive accumulation of drugs into target tissue due to the method or site of administration or employs the specific tissue property mainly found in tumors, that is called the enhanced permeability and retention (EPR) effect. This is described by the nature of leaky blood vessels in the tissue accompanied by the growing tumor's increased uptake of nutrients and oxygen from the blood [42]. In contrast to passive targeting, active targeting relies on tissue environment-specific accumulation or the usage of specific targeting affinity ligands.

1.4.1 Cell-penetrating peptides

Cell-penetrating peptides (CPP) are relatively short, typically 4–40 amino acids long peptides, that conventionally carry a positive net charge. They are capable of effectively crossing cell membranes and also transporting biologically active cargo molecules inside a wide variety of cells in vitro and in vivo. The delivered payload can largely vary from small molecule drugs and oligonucleotides to peptides, proteins, nanoparticles and imaging agents [43]. The advantages of CPPs are that they are relatively easy to synthesize and modify, whereas the solid phase peptide synthesis ensures their sequence-defined structure and monodispersity [44]. CPPs are highly biocompatible and therefore have proven to be relatively non-toxic to cells and non-immunogenic in preclinical mouse models [45].

1.4.1.1 History and classification

The first recordings of a protein being able to cross cellular membranes was published in 1988. It was the transactivator of transcription (Tat) protein from HIV-1 that localizes to the nucleus after cellular uptake [46,47]. The next CPP discovered in 1991 was the Drosophila antennapedia homeobox peptide [48], followed by identification of the same peptide's minimal functional cell-penetrating part in 1994, called penetratin [49]. In 1998, the first chimeric CPP that combines the sequences from two different naturally occurring proteins, was designed. More specifically, mastoparan peptide from wasp venom was fused to the fragment of human neuropeptide galanin by a lysine residue and the resulting chimeric peptide was called transportan [50]. The very first fully synthetic CPP, the model amphipathic peptide (MAP or KLAK), was also designed in 1998 [51].

The rapidly increased amount of new CPPs designed differ from each other in terms of their amino acid composition, polarity, hydrophobicity, length, charge, etc. The basis for their classification can be very different [52]. From the previous paragraph it is possible to see three classes of CPPs based on their origin: natural protein-derived, purely synthetic or chimeric CPPs. Another option for classification into three groups can be based on the peptides' physicochemical properties: their charge, hydrophobicity and amphipathicity – the positioning of positively charged and/or hydrophilic and hydrophobic peptide regions into opposite

side of the molecule. This strategy enables the interaction of the cationic part with the negatively charged cell membrane and at the same time the hydrophobic region is responsible for the insertion into the lipid membrane. The amphipathic CPPs are the most numerous ones. The last alternative for classification is on the basis of their conformation: linear versus cyclic. Only approximately 5% of the CPPs are cyclic, but this structure provides several advantages: lower sensitivity to proteolytic degradation and higher cell permeability, presumably due to the higher affinity with the target receptor on the cell surface [53].

1.4.1.2 Cellular uptake mechanisms

The uptake mechanism of the CPPs is not universal, they can enter cells via two main mechanisms – by either the endocytic pathways or via direct translocation (Figure 7).

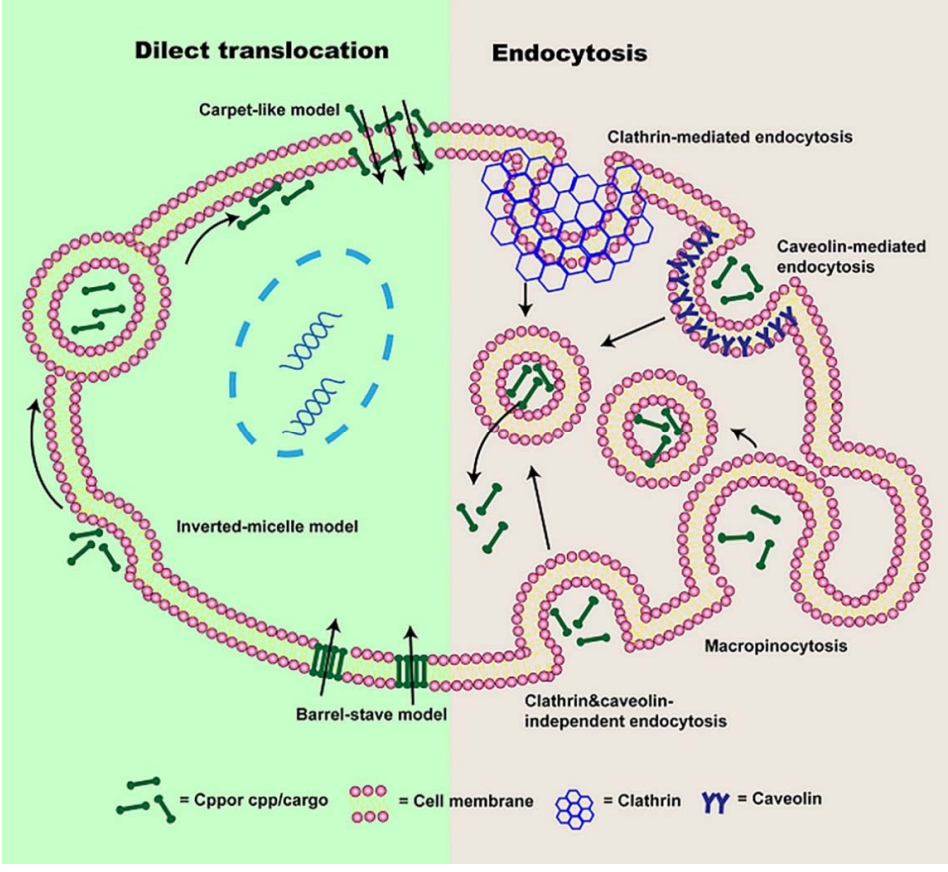

Figure 7. The schematic illustration of cellular uptake mechanisms of CPP or CPP/cargo. Reprinted from Xie et al [53].

Endocytosis can be subdivided according to the specific proteins that are responsible for the endosome formation: clathrin- or caveolae-mediated endocytosis, macropinocytosis, and clathrin- and caveolae-independent endocytosis. The specific route is dependent on the properties of the peptide and its concentration, of the cargo molecules and the cell type being transfected [54]. Although some hydrophobic CPPs have been shown to be able to translocate directly across cell membranes into the cytoplasm [55], the main entry mechanism is still thought to be endocytosis. Nanoparticles on the other hand are predominantly incapable of direct diffusion through the cell membrane due to their size and surface charge, therefore they require active transport. The most prevalent internalization pathway for NPs is considered to be clathrin-mediated endocytosis [56].

1.4.1.3 CPP modifications

Similarly to other non-viral vectors, one of the largest limitations in CPP and its cargo delivery is their entrapment into the endosomes and subsequent lysosomal degradation. There are various chemical modifications which can be introduced to the peptide sequence that could resolve the issue and help the endosomal escape of the peptide or its nanoparticles. This also gives the opportunity to use smaller amounts of NPs and increase the transfection efficacy [57]. One of the best examples for this kind of CPPs are the transportan10 (TP10) [58] derived families of PepFects (PF) and NickFects (NF) [59–61]. First, all of them have a fatty acid modification in the N-terminus, most commonly a stearic acid. The addition of a hydrophobic moiety of such kind has been shown to facilitate endosomal escape, enhance nucleic acid binding to CPP and the stability of the nanoparticles [62] and most importantly, mediate efficient ON delivery in vivo [63].

The two CPPs mainly used and/or designed in this thesis are highly efficient CPPs for siRNA delivery in vitro and in vivo, PepFect6 (PF6) [59] and NickFect70 (NF70) [64]. Both of them have additional modifications that promote endosomal release of the nanoparticles. PF6 has chloroquine analogs covalently attached via a succinylated lysine tree and NF70 has histidine residues in the peptide sequence. Both CPPs take advantage of the proton sponge effect: the chloroquines or histidines become protonated in the acidic environment of the endosome, induce osmotic swelling and eventually endosomal rupture. The protonation also induces the conformational change of the peptides to α-helical structures that promote the endosomal escape by interacting with the endosomal membrane [65]. The chloroquines as well as some other chemical agents are also capable of disrupting the endosomal membrane. In addition, several modifications that are inspired by viruses or bacterial toxins can aid endosomal escape, like the addition of fusogenic or endolytic proteins or their endosomal escape promoting domains [66].

Another frequently used modification is the conjugation of a polyethylene glycol (PEG) molecule to the CPP or NPs. This strategy is also clinically approved and it is used in order to increase the serum stability – avoid enzymatic degradation, opsonization and the immune activation – which prolongs the circulation

time. As a hydrophilic polymer, PEG also increases the solubility of hydrophobic drugs. The drawbacks of PEGylation are that it reduces the interactions to cell surfaces which also inhibits cellular uptake by the target cells. PEG has also been shown to decrease endosomal escape which on the other hand reduces the desired therapeutic efficiency [67].

The advantage of CPPs being highly efficient in transfecting a large variety of cells in vitro and in vivo can on the other hand be a major drawback. If a drug has to reach its specific target tissue in vivo, the lack of specificity of CPP-mediated delivery leads to the loss of material to other tissues. In case of systemic administration, the NPs mostly accumulate non-specifically to the first pass tissues, lungs and liver, which can induce toxic effects [68]. To overcome this issue, it is possible to functionalize CPPs with various targeting moieties like antibodies or their fragments or targeting peptides further described in the next chapter.

Another possibility is to use activatable CPPs, in which case the cell-penetrating activity is masked by a polyanionic or hydrophilic inhibitory domain, e.g., PEG, that is covalently attached via an environment-sensitive linker [69]. The shielding moiety will be cleaved from the CPP once the NPs reach the suitable environment present only in the target tissue. The linkers can be sensitive to specific proteases, for example to matrix metalloproteinases (MMPs) that have been shown to be upregulated in tumor [70,71] and endometriosis [72] tissues, since they are vital for the degradation of extracellular matrix during invasion, migration and angiogenesis. The acidic pH in the target tissues [73,74] compared to the physiological neutral pH in the normal tissues and the blood can also be harnessed for active drug delivery. One option is that activatable CPPs can be shielded using a pH-cleavable linker. It is also possible to include histidines in the CPP sequence that have neutral charge and are hydrophobic in neutral pH environment, but become hydrophilic in acidic conditions and cationic due to protonation, which leads to pH-sensitive tissue accumulation [65].

1.4.2 Systemic homing peptides

Many previous *in vivo* preclinical cancer research studies have depended exclusively on the passive accumulation of therapeutic candidate molecules into tumors by EPR effect. Recently, there have been studies suggesting that the EPR effect might likely be present in endometriosis lesions as well [75]. The potential drawback is that this approach is not specific and solely relies on the nanoparticle passive accumulation into the lesions without any active targeting, which means that there is a possibility of the nanoparticles affecting healthy tissues as well. This is supported by the fact that in most cancer therapy clinical trials, therapeutic candidates without active targeting are not proving to be more successful when comparing to conventional chemotherapy [76,77]. It has also been shown that solid tumor permeability can vary extensively between patients and even in different tumors of an individual patient [78,79].

In addition to CPP modifications described in the previous chapter, another possibility is to combine CPPs with specific affinity ligands, called homing or targeting peptides, that selectively bind to specific tissues or cells. One of such examples has been published by Langel's group, a glioma-targeting peptide gHoPe2 [80]. In addition to CPPs, targeting peptides are also capable of guiding various other molecules to selected tissues, e.g., different types of polymers, therapeutic NPs or diagnostically detectable molecules like radionuclides [81]. Their advantages over other affinity ligands, for example antibodies is that targeting peptides have a small size, high stability, low immunogenicity and they can also be chemically modified, while preserving their targeting ability [82].

Teesalu laboratory uses in vivo screening of phage libraries in live mice to identify systemic homing peptides that specifically target different normal and diseased tissues, most importantly solid tumors. The power of in vivo phage screening is illustrated by discovery of internalizing RGD (iRGD) peptide and other tumor penetrating peptides (TPP). These peptides activate an endocytic transport pathway related to but distinct from macropinocytosis through a three-step process that involves binding to a primary, tumor-specific receptor, a proteolytic cleavage, and binding to a second receptor, neuropilin-1 (NRP-1) to activate the transport pathway. This trans-tissue pathway, dubbed the CendR pathway, mediates the extravasation and transport through tumor tissue of payloads ranging from small molecule drugs to nanoparticles. Targeting with TPP has been shown to specifically increase the accumulation of NPs in experimental tumors in vivo, and in human tumors ex vivo. The critical element in all TPPs is the presence of the cryptic R/KXXR/K position-dependent motif able to interact with cell and tissue penetration receptor NRP-1 only when exposed at the C-terminus. The strict requirement of C-terminal exposure prompted to refer to the motif as the C-end rule (CendR) motif [83]. CendR receptor NRP-1 is widely expressed in normal tissues and overexpressed on the endothelial cells and in malignant and malignancy-associated cells in a wide range of solid tumors, including prostate, breast and pancreas carcinoma, melanoma, and glioblastoma [84,85]. Whereas TPPs use different primary recruitment receptors, such as αv integrins for uCendR and iRGD [86,87], p32/gC1qR for LinTT1 [88], and CD13 for iNGR [89], they all converge at using CendR-based mechanism for tumor penetration. Currently, iRGD peptide is in clinical development for treatment of patients with metastatic pancreatic ductal adenocarcinoma. In phase 1 clinical trial, iRGD (co-administered with standard of care nab-paclitaxel and gemcitabine) demonstrated an acceptable safety profile with no dose-limiting toxicities and encouraging activity in patients [90]. iRGD is currently undergoing a phase 2b testing (ClinicalTrials.gov Identifier: NCT05042128) [91]. A multitude of other tumor homing peptides have been developed over the years to allow for targeted drug delivery with great precision to various components in malignant tissues: blood and lymphatic vascular trees, extracellular matrix, immune cells and fibroblasts. Homing peptides are typically not species-specific as they target functionally important binding pockets on target molecules, and these sites are highly conserved among species. Compared to antibodies with nanomolar dissociation

constants (KD), the affinities of TPPs to their receptors are lower, thereby avoiding the "affinity site barrier" for better extravasation and deeper penetration into tumor parenchyma. Translational potential of homing peptides is further strengthened by their small size compared to antibodies, low immunogenicity and biocompatibility.

In addition to numerous novel therapeutic molecules suggested in the literature for the treatment of endometriosis, Salumets's group has also proposed several compounds [92,93] and microRNAs [94] that could be used in endometriosis therapy. However, there are no tools available for precision delivery of therapeutic compounds into the endometriosis lesions inside a living organism. Identification of a specific targeting peptide for endometriosis could help to overcome the issue of unspecific accumulation of therapeutics in healthy organs. The specific delivery of therapeutic molecules would also accelerate the development of nanoparticle-based approaches for endometriosis and help to protect the payloads from degradation in plasma, to encapsulate several types of therapeutic molecules and to improve pharmacokinetics. Furthermore, it would enable the development of diagnostic tools, e.g., magnetic particles for non-invasive diagnosis of endometriosis using MRI or PET.

1.4.2.1 Phage display

Different targeting peptides are discovered by using in vitro, ex vivo, or in vivo phage biopanning methods [95]. In vivo phage display has become a widely used high-throughput tool for the identification of homing peptides that takes the complexity and heterogeneity of living animals into account [96]. It enables the mapping of systemically accessible molecular patterns of normal organs and diseased tissues. This technology has enabled assessment of the extent of the molecular specialization in the vasculature and has contributed to identification of a number of new markers expressed in the tumor neovasculature.

For biopanning, T7 bacteriophage libraries displaying about a billion of random genetically encoded cyclic peptides as fusions to phage coat protein are injected into experimental animals or incubated directly with the tissue of interest (Figure 8). The size of T7 phage and the covering peptide density are similar to typical clinical nanoparticles. This increases the potential of the discovered peptides' efficacy in mediating NP homing in preclinical settings. The cyclization constrains the peptide conformation, which increases the interaction with the target receptor [97]. After biopanning, the phages that display peptides which bind with higher affinity to the tissue are rescued and the biopanning steps are repeated for the enrichment of the targeting peptide. Eventually, the displayed peptides are subjected to next-generation sequencing (NGS) for identification.

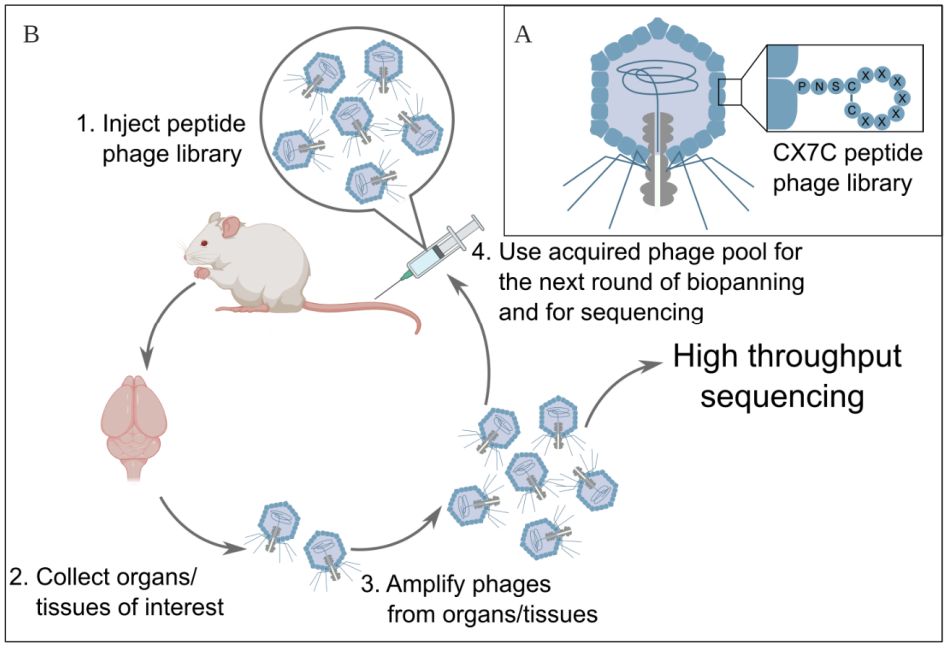

Figure 8. Representation of in vivo phage display biopanning. Reprinted with permission from Põšnograjeva et al., Copyright © 2022, The Author(s), under exclusive license to Springer Science Business Media, LLC, part of Springer Nature [98].

1.4.2.2 Targeting extracellular proteins

In cancer research, the importance of extracellular matrix (ECM) composition and structure in the tumor microenvironment has been widely studied. During tumor formation, the cancer cells direct the ECM to increase its stiffness by increasing the production of ECM proteins. In return, the increased ECM stiffness induces further malignant transformation. Furthermore, the ECM also influences the vascularization and the cancer cells' adhesive, migrative and proliferative properties [99]. As a result, targeting the dysregulated cancer ECM proteins is considered a highly promising strategy for influencing the malignant cells themselves. This is supported by the recent discoveries by Prof. Teesalu's group, where they developed a series of homing peptides that target overexpressed cancer ECM proteins. More specifically, fibronectin Extra Domain-B (Fn-EDB) and tenascin C C-isoform (TNC-C) binding PL1 peptide [100], Fn-EDB and NRP-1 binding PL2 [101] and TNC-C and NRP-1 binding PL3 peptide [102]. When administering the PL1- and PL3-guided therapeutic nanoparticles into tumor-bearing mice, the tumor progression was reduced and mouse survival increased [100,102]. In contrast to mono-specific targeting ligands, PL1 and PL3 peptides engage with two target molecules. As the expression of the peptide receptors in the tumor tissue is heterogeneous, simultaneous targeting of two receptors "averages" the peptide accumulation over tumor tissue resulting in

more uniform distribution of payloads. This is an important aspect, as heterogeneity in anticancer payload accumulation is known to lower the therapeutic efficacy, and eventually, lead to acquisition of therapeutic resistance. Besides selective homing to malignant ECM, PL1 and PL3 can trigger endocytic uptake in the malignant cells. Cell internalization can dramatically expand the range of therapeutic payloads to intracellularly-acting anticancer agents and radionuclides, including different anticancer drugs.

TNC-C, Fn-EDB and also Fn-EDA are all normally expressed during embryogenesis, wound healing processes and in the perivascular area around the angiogenic blood vessels, but their expression is not present in normal healthy adult tissues [103,104]. As well as cancer, endometriosis is also considered an angiogenic disease, that has neovascularized areas around the lesions [37]. The expression of tenascin and fibronectin has been shown to be upregulated in endometriotic lesions [24]. In addition, a Fn-EDA-targeting antibody was shown to effectively accumulate to endometriotic lesions of an in vivo mouse model [105].

1.5 Nanomedicine

1.5.1 Desired nanoparticle characteristics

To increase the translational potential of nanoparticles, various requirements have to be met. The NPs have to be biocompatible and bioavailable, stable, non-toxic and non-immunogenic, and the production has to be cost-effective. The key physicochemical characteristics that need to be optimized during nanoparticle development are their size, charge and hydrophobicity.

To enhance the bioavailability, the aimed NP size range falls between 10–100 nm, which promotes saturation solubility and also longer circulation times [106]. The critical size limit is 5–200 nm; particles under 5 nm are cleared from the organism relatively quickly by the kidneys and NPs above 200 nm accumulate in the liver and spleen. The large particles are also likely to be entrapped in the lung capillaries, which can induce toxic effects [107]. The biodistribution and efficacy of NPs is highly dependent on their surface charge. The most successful NPs are usually slightly negatively or slightly positively charged. Although cationic NPs are efficient in crossing negatively charged cell membranes, the high positive charge can lower the circulation time, induce toxicity and nonspecific uptake [108].

The main advantage of loading drugs into NPs is that this enables the stabilization of the drug or oligonucleotide and increases the shelf life of the therapeutics. It also prolongs the circulation time and on the other hand often allows the reduction of the drug dose, which helps to reduce the off-target side effects. Many of the recently developed drugs present substantial lipophilicity and high molecular weight. The encapsulation into NPs increases bioavailability by aiding the delivery of hydrophobic and poorly water-soluble drugs, for example most

chemotherapeutics. In addition, compared to the systemic administration of conventional chemotherapeutics, their packaging into NPs and passive targeting by exploiting the EPR effect significantly reduces their toxicity to normal tissues [106].

1.5.2 Non-covalent nanoparticles

The NPs can be formed either in a covalent or non-covalent manner. Covalent conjugation is based on chemical crosslinking. While it results in more stable and well-defined complexes, the non-covalent strategy is less laborious and is considered more suitable for larger charged molecules like nucleic acids. CPPs enable quick and simple non-covalent peptide/ON nanoparticle formation (Figure 9) [43]. The NPs are forming spontaneously in aqueous environment due to electrostatic and hydrophobic interactions of the negatively charged nucleic acid and cationic peptide. This phenomenon was first described in 1997 and since that has been exploited widely for nucleic acid delivery [109].

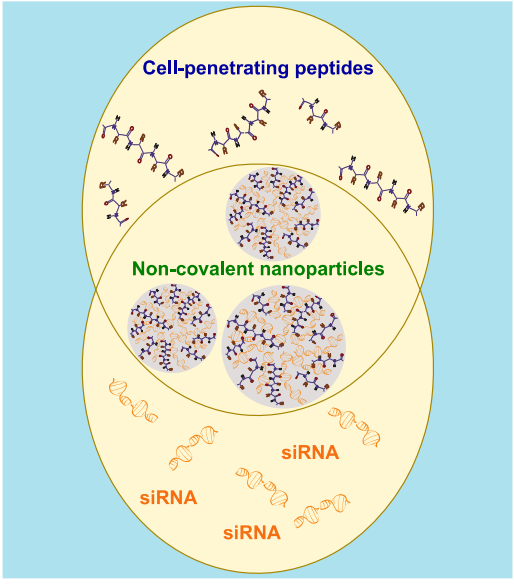

Figure 9. Schematic representation of the forming non-covalent nanoparticles.

1.5.3 Silver nanoparticles

Metallic NPs are generally stable and biocompatible, falling within the 1–100 nm size range and they can be relatively easily synthesized and modified. This enables the decoration of their surface with various molecules like fluorescent probes, drugs or targeting moieties. The fluorophore-coated metallic carriers have amplified fluorescent signals due to plasmonic enhancement [106]. Silver nanoparticles (AgNPs) are widely used for medical purposes e.g., in wound dressings, dental implants, during imaging and for their antibacterial properties in medical device coatings, also in cosmetics, textile industry etc [110]. One of the main advantages of using AgNPs in preclinical in vitro and in vivo studies is the option of distinguishing the membrane-bound extracellular NPs from the internalized fraction. This is achieved by using a biocompatible etching solution [111].

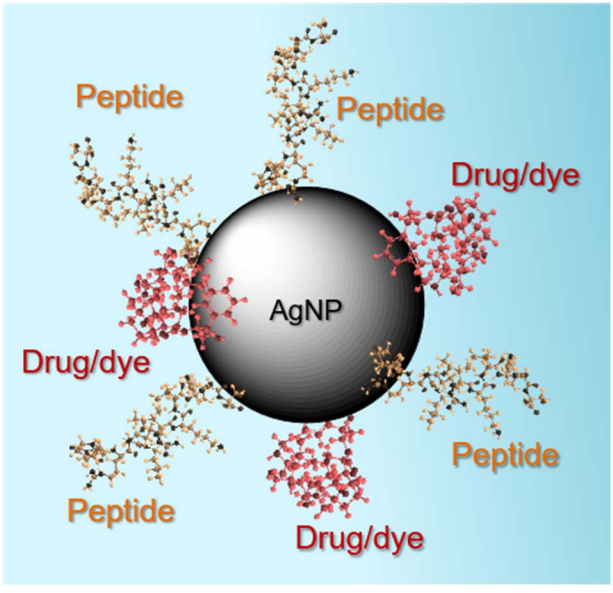

Figure 10. Schematic representation of the used silver nanoparticles.

1.5.4 NP-based applications in medicine

The first medically used nanoparticle Diprivan®, a general anesthetic propofol loaded into liposomes, was approved by the US Food and Drug Administration (FDA) already back in 1989. In 1995, FDA approved the first nanodrug Doxil®, PEGylated liposomal doxorubicin, for the treatment of several cancers. NP-based therapeutics have been used for various types of conditions, for example decreased vision, fungal infection, hypertension, osteoarthritis, multiple sclerosis and mainly for various cancers [106].

In August 2018, the RNAi research field reached the ultimate goal, when the FDA and European Medicines Agency (EMA) approved the first RNAi-based drug, patisiran (ONPATTRO™) – a double-stranded siRNA encapsulated in a lipid nanoparticle acting on the liver hepatocytes. It is used for the treatment of hereditary transthyretin amyloidosis with polyneuropathy [112].

Just recently, in September 2022, the FDA approved the first and only CPP-formulated drug, DAXXIFY™ [113]. A neuromodulator DaxibotulinumtoxinA for Injection (DAXI) is a novel botulinum toxin type A formulation for the treatment of moderate or severe glabellar lines and a number of neurologic and musculoskeletal conditions. The formulation consists of a 35 amino acids long stabilizing peptide RTP004 that has a high cationic charge at physiologic pH and forms a strong electrostatic bond with the highly purified neurotoxin DAXI [114].

Except for some antibody-functionalized drugs [115], there are no active drug delivery options available and most of the approved formulations have been relying on the passive targeting [106]. There are no approved targeting peptide-based drug carriers available, most of the formulations have been polymeric or lipid nanoparticles by their nature. The notable COVID-19 vaccines by Pfizer/BioNTech and Moderna are also PEGylated liposomal nanoparticles encapsulating the mRNA encoding viral protein [116].

2. AIMS OF THE STUDY

The objective of the study was to develop or repurpose peptide-based drug carriers for various nanomedical applications. The formulations of different peptides were tested in several preclinical disease models, with main focus on endometriosis.

The specific aims were as follows:

Paper I to rationally design a pH-sensitive delivery vector that efficiently transports siRNA into cells both in vitro and in vivo. A series of different peptide modifications and their transfection performance was studied.

Paper II to test the potential of CPP/siRNA nanoparticles for cancer and endometriosis therapy. PepFect6 and NickFect70 were used as delivery vectors for therapeutic siRNAs against RRM2 and VEGF and their efficacy was tested in monolayer and 3D spheroid cell cultures.

Paper III to investigate the therapeutic potential of homing peptide-based targeting of endometriosis. The effect of targeting the extracellular proteins Fn-EDB and TNC-C with PL1-AgNPs was studied in 2D and 3D cell cultures and clinical endometriosis lesions.

3. METHODOLOGICAL CONSIDERATIONS

The detailed description of the materials and methods used in the current thesis are presented in the corresponding original publications. Only a brief overview of the selected methods is provided in this chapter.

All animal experiments and procedures conducted were approved by the Estonian Laboratory Animal Ethics Committee.

The Research Ethics Committee of University of Tartu approved the patient sample collection and the informed consent forms, which were signed by all the participants.

3.1 Peptide design and synthesis

The sequences of all used peptides are presented in Table 1. The homing peptides used in Paper III were ordered from a commercial supplier (TAG Copenhagen, Denmark). All CPPs (Paper I and II) were synthesized using standard protocols for the fluorenylmethyloxycarbonyl (Fmoc) solid phase peptide synthesis strategy that was developed already in the year 1963 [121]. The forming peptide chain is covalently attached to a solid phase, called resin, that remains stable during all chemical reactions during synthesis. The approach relies on a stepwise addition of amino acids with protected α-amino groups, which is later removed under basic conditions. The peptide removal from the resin on the other hand is carried out under acidic conditions. In this work, rink-amide ChemMatrix resin was used to obtain C-terminally amidated peptides. As coupling reagents, HOBT/HBTU in DMF and DIEA as an activator base were used. The fatty acid was coupled manually to the N-terminus of the peptide. Peptides were purified by high-performance liquid chromatography (HPLC) by using acetonitrile/water gradient containing 0.1% TFA. The molecular weight of the peptides was analyzed by MALDI-TOF mass spectrometry. The concentration of the peptides was determined based on dilutions of accurately weighed substances and absorption of tyrosine, where applicable.

Table 1. Peptide sequences used in this thesis

CPP	Sequence	Paper	Reference
NF55	stearyl-AGYLLGO[a]INLKALAALAKAIL-NH_2	I	[117]
PF6	stearyl-AGYLLGK[b]INLKALAALAKKIL-NH2	I, II	[59]
NF71	stearyl-HHYHHGO[a]ILLKALKALAKAIL-NH2	I	Paper I
NF712	arachidyl-HHYHHGO[a]ILLKALKALAKAIL-NH2	I	Paper I
NF713	behenyl-HHYHHGO[a]ILLKALKALAKAIL-NH2	I	Paper I
NF700	HHHHYHHGO[a]ILLKALKALAKAIL-NH2	I	Paper I
NF701	decanyl-HHHHYHHGO[a]ILLKALKALAKAIL-NH2	I	Paper I
NF702	stearyl-HHHHYHHGO[a]ILLKALKALAKAIL-NH2	I	Paper I
NF70	arachidyl-HHHHYHHGO[a]ILLKALKALAKAIL-NH2	I, II	Paper I
NF703	behenyl-HHHHYHHGO[a]ILLKALKALAKAIL-NH2	I	Paper I
NF704	stearyl-HHHHHHGO[a]ILLKALKALAKAIL-NH2	I	Paper I
NF705	arachidyl-HHHHHHGO[a]ILLKALKALAKAIL-NH2	I	Paper I
NF706	behenyl-HHHHHHGO[a]ILLKALKALAKAIL-NH2	I	Paper I
NF707	stearyl-HHHHHHYLLGO[a]ILLKALKALAKAIL-NH2	I	Paper I
NF708	arachidyl-HHHHHHYLLGO[a]ILLKALKALAKAIL-NH2	I	Paper I
NF709	behenyl-HHHHHHYLLGO[a]ILLKALKALAKAIL-NH2	I	Paper I
NF721	stearyl-HHHHHHYHHGO[a]ILLKALKALAKAIL-NH2	I	Paper I
NF72	arachidyl-HHHHHHYHHGO[a]ILLKALKALAKAIL-NH2	I	Paper I
NF722	behenyl-HHHHHHYHHGO[a]ILLKALKALAKAIL-NH2	I	Paper I
PL1	PPRRGLIKLKTS	III	[100]
PL2	TSKQNSR	III	[101]
PL3	AGRGRLVR	III	[102]
RPAR	RPARPAR	III	[83]
iRGD	C[c]RGDKGPDC[c]	III	[118]
EM1	VRRADNRPG	III	[119]
EM2	RTRLHTR	III	[120]

[a] Synthesis continued from the sidechain amino group instead of α-amino group; [b] Four trifluoromethylquinoline moieties attached to succinylated lysine tree; [c] Disulfide bond.

3.2 Nanoparticle formation

3.2.1 Non-covalent formulation strategy

All CPP/siRNA NPs used throughout the thesis were formed in a non-covalent manner. The used siRNA sequences are listed in Table 2. The particles were formed by mixing the peptide with siRNA in MilliQ water (pH 5.3–6.3) using peptide to siRNA molar ratios (MR) up to MR40. The mixtures were incubated at room temperature (RT) for 45–60 min and directly thereafter the nanoparticles were used for subsequent experiments. As negative controls, siRNA without a delivery vector at equivalent concentrations to nanoparticle transfection was added to experiments. In many experiments throughout the work, commercially available Lipofectamine™ RNAiMAX Transfection Reagent was used according to manufacturer's protocol as a positive control for siRNA delivery.

Table 2. siRNA sequences used in this thesis

siRNA	Strand	Sequence	Paper
siLuc	Sense	5'-ACGCCAAAACAUAAAGAAAG-3'	II
	Antisense	5'-UUCUUUAUGUUUUUGGCGUCU-3'	
siLuc2	Sense	5'-Cy5-GGACGAGGACGAGCACUUCtt-3' [a]	I
	Antisense	5'-GAAGUGCUCGUCCUCGUCCtt-3' [a]	
siLuc2_Cy5	Sense	5'-Cy5-GGACGAGGACGAGCACUUCtt-3' [a]	II
	Antisense	5'-GAAGUGCUCGUCCUCGUCCtt-3' [a]	
siGFP	Sense	5'-GGCUACGUCCAGGAGCGCACC-3'	I
	Antisense	5'-UGCGCUCCUGGACGUAGCCUU-3'	
siFVII	Sense	5'-GGA(2′F-U)(2′F-C)A(2′F-U)(2′F-C)(2′F-U)(2′F-C)AAG(2′F-U)(2′F-C)(2′F-U)(2′F-U)a(2′F-C)tt-3' [a]	I
	Antisense	5'-G(2′F-U)AAGA(2′F-C)(2′F-U)(2′F-U)gaga(2′F-U)ga(2′F-U)(2′F-C)(2′F-C)tt-3' [a]	
siRRM2	Sense	5'-GAUUUAGCCAAGAAGUUCAGAUUac-3' [a]	II
	Antisense	5'-GUAAUCUGAACUUCUUGGCUAAAUCUU-3'	
siVEGF	Sense	5'-AUGUGAAUGCAGACCAAAGAAtt-3' [a]	II
	Antisense	5'- UUCUUUGGUCUGCAUUCACAUtt-3' [a]	

[a] Lowercase letters refer to deoxynucleotides.

3.2.2 Silver nanoparticle synthesis and functionalization

The silver nanoparticles used in Paper III were synthesized according to the citrate method [122] and functionalized as previously described [111,123]. Briefly, biotinylated peptides were used as the targeting component and attached to the NeutrAvidin moiety of the AgNP. The avidin-biotin interaction is an unusually strong non-covalent bond occurring in nature that is often utilized in biotechnological applications [124]. The NHS-functionalized forms of the fluorophore CF555 or the therapeutic payload monomethyl auristatin E (MMAE) linker were coupled to the terminal amine group of polyethylene glycol (PEG) on the AgNP. The control particles were prepared by blocking a biotin-binding pocket with free D-biotin.

3.3 Physicochemical characterization of nanoparticles

3.3.1 Dynamic light scattering

Dynamic light scattering (DLS) is often used as a first step in the characterization of NP physicochemical properties that confirms the successful formation of nano-sized particles. DLS is a fast, precise, and reproducible quality control method to check the homogeneity and aggregation of the biomolecules present in a sample [125]. DLS measures the hydrodynamic diameter of the particles in aqueous solutions. For this study, the Zetasizer apparatus was used which also provides the possibility of determining the zeta potential of the molecules that correlates with the particles' surface charge. The two parameters, size and surface charge, are one of the most important ones influencing the nanoparticles' cellular uptake and in vivo biodistribution.

3.3.2 Transmission electron microscopy

DLS gives informative size distribution data of the nanoparticles, but does not provide any information of the NPs' morphology and this is where transmission electron microscopy (TEM) becomes important. The significant difference compared to DLS is that in case of TEM the samples are not analyzed in aqueous environment, but were absorbed on a carbon film-covered side of a copper grid and this provides the opportunity for morphological visualization. TEM has the same concept as a regular light microscope but it uses a high energy focused beam of electrons instead of light. TEM provides extremely high magnification and resolution compared to light microscopy since the wavelength of electrons is much smaller than that of the light [126].

3.3.3 Heparin displacement assay

When thinking about the further goal of using NPs for therapeutic or diagnostic purposes, the NPs have to be stable enough to resist the primary degradative or neutralizing blood components in order to achieve desired functional changes [127]. The heparin displacement assay (HDA) allows the evaluation of the strength of the CPP/siRNA particles. Heparin sulfate has high negative charge density and it is capable of displacing nucleic acids from labile nanoparticles. Different concentrations of heparin needed for the dissociation of NPs show dose-dependent particle stability. The HDA results were measured by quantifying the fluorescence of nucleic acid dye PicoGreen bound to the free or released siRNA.

3.3.4 Resistance to enzymatic degradation

Protease susceptibility is one of the main disadvantages of CPP-guided nanoparticles[128]. Therefore, a proteolytic degradation assay was performed to evaluate the nanoparticles' resistance against degradation. For that, preformed CPP/siRNA NPs were exposed to Proteinase K treatment and the amount of released siRNA over different timepoints was measured with PicoGreen assay.

3.4 Cellular feasibility studies

The primary proof-of-concept biological functionality testing of different drugs often starts with conducting cell and tissue culture experiments. Therefore, the theranostic potential of previously described peptide-based drug carriers and their nanoparticles was further investigated in a series of various cell culture and ex vivo experiments. The list of all the used cells is presented in Table 3.

All cell cultures were maintained at 37 °C, 5% CO2 in a humidified environment with appropriate growth media supplemented with serum and antibiotics. For the formation of 3D spheroids, two previously described methods were used. Namely, in Paper II, the cells were seeded on low-attachment 96U-well plates [129] and in Paper II the spheroids were formed by using the magnetic 3D bioprinting method [130].

Table 3. Cells used in this thesis

Type	Name	Origin	Reporter expression	Paper
Cell line	U-87MG	Human glioblastoma	–	I, II
	U-87MG-Luc2	Human glioblastoma	Luciferase 2	I
	CHO-GFP	Chinese hamster ovary	Green fluorescent protein	I
	HT1080	Human fibrosarcoma	–	II
	12Z	Human EMS epithelium	–	III
	HESC	Human endometrium stroma	–	III
Primary cells	RBC	Murine red blood cells	–	I
Primary culture	Peritoneal stromal cells	Human peritoneal endometriotic lesion	–	II
	Ovarian stromal cells	Human ovarian endometrioma	–	II

3.4.1 Hemolytic activity assay

Red blood cells (RBC) have previously been used for ex vivo analysis of the membrane activity and toxicity and also for the evaluation of endosomolytic properties of pH-responsive biologic drugs [18]. Membrane activity of peptides was evaluated after incubation of murine RBCs with

CPP solutions at 37 °C. The released hemoglobin was quantified by measuring absorbance of the supernatant at 540 nm. Since the NickFects are pH-sensitive CPPs, a modified version of the same hemolysis quantification was conducted. The endosomolytic activity of the peptides was thereby assessed at different buffer pH conditions.

3.4.2 Peptide-phage cell binding

In Paper III, in order to reveal peptides that specifically bind to endometriosis cells, T7 bacteriophages were prepared. To generate a phage that expresses a peptide of interest on its surface, nucleotide sequence coding for that peptide was inserted into the phage's genomic DNA in frame with the major capsid protein. Different previously discovered homing peptide expressing phage clones were amplified in complementing host bacterial cells BLT5403 and purified with polyethylene glycol according to an established protocol [131]. The retrieved phage concentration was measured and a defined amount of 5×10^7 PFU/mL of phage was thereafter incubated with the detached endometrial cells at 4 °C for 1 h. Subsequently, the cells were washed to remove the unbound phages and the bound phages were quantified by titering and the plaque assay [98].

3.4.3 Nanoparticle cell internalization

For testing the AgNP uptake in endometrial cells in Paper III, the CF555-labeled AgNPs were applied on growing cells and incubated at 37 °C for 2 h. It is possible to dissolve the particles that have not internalized and are only bound to the surface of the cells by washing with a mild biocompatible etching solution consisting of $K_3Fe(CN)_6$ and $Na_2S_2O_3$. The cells were subsequently detached and subjected to flow cytometry for CF555 detection of the internalized NPs.

3.4.4 Reporter gene assay

For assessing the functional efficacy of NPs in Paper I, the reporter-expressing cell lines U87-Luc2 or CHO-GFP were transfected with CPP/siLuc2 or CPP/siGFP NPs, respectively. The transfection efficacy was quantified 24 h later for Luc2 or 48 h later for GFP, depending on the half-life of the target gene. The luciferase detection-based reporter systems are highly sensitive and specific. The results reflect the smallest changes in transfection levels and also enable the evaluation of biological activity. The fluorescence-based reporter systems like e.g., using stable GFP expression as siRNA target also mirrors biological activity of NPs, but on the other hand allows the detection of reporter gene downregulation by confocal microscopy or FACS to quantify the number of successfully transfected cells.

3.4.5 Therapeutic gene knockdown

In Paper II, in order to validate the therapeutic siRNAs and optimize the transfection conditions, gene expression levels were quantified first in tumor, then in endometriotic primary cells and eventually in 3D spheroids of both cell types. 48 h after transfection, total mRNA was isolated from the cell cultures and cDNA was subsequentially synthesized. Thereafter the gene expression levels were measured using quantitative real time PCR (qRT-PCR). This PCR method combines the amplification of the target DNA with the detection steps, therefore there is no need for post-PCR sample handling. The advantages of qRT-PCR include high reproducibility and accuracy in addition to the high-throughput potential of the assay [132].

3.4.6 Immunocytochemistry

Immunocytochemistry (ICC) and immunohistochemistry (IHC) are one of the most frequently used techniques in life sciences. The immunostainings can provide crucial information about the localization of different cells and proteins in tissue sections or even visualize high-resolution intracellular compartments. In addition, it is possible to stain several markers on one sample. The expression of different proteins was investigated in Papers II and III. For that, the cells were first transfected (in Paper II only) and 48 h later fixed with PFA. This was followed by the primary antibody incubation in blocking buffer containing the serum of host animal of the secondary antibody. Thereafter the fluorescently labelled secondary antibody and DAPI dye staining the nuclei were applied on the cells. The fluorescence of the dyes was visualized under confocal microscope.

3.4.7 Cell cycle analysis

Cell cycle analysis was performed after the treatment with CPP/siRRM2 NPs, since RRM2 knockdown is known to inhibit cell proliferation. For that, tumor or endometriotic cells were transfected, detached 48 h later and fixed with ethanol. Cell cycle is usually determined by the amount of DNA present in the cells. This is done by staining the cells with a fluorescent dye called propidium iodide (PI) that is binding between the base pairs of DNA. Since PI is also capable of binding to RNA, the RNase A was added to the PI staining solution. The cell cycle was eventually analyzed using flow cytometry.

3.4.8 Cytotoxicity evaluation

There is a large variety of commercially available viability or cytotoxicity assays. In this work, two kits from Promega were used to determine the viability of cells after different types of treatments. More specifically, in Paper I, the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) was used to measure the viability after treating with CPP/siRNA NPs or free peptides at different concentrations. Thereafter the absorbance of the formed formazan product was quantified.

In Paper III, the viability was measured for another purpose – to evaluate the efficacy of a cytotoxic antimitotic drug MMAE covalently bound to PL1-AgNPs. For that purpose, the CellTiter-Glo® Luminescent Cell Viability Assay was used 48 or 72 h after the treatment of endometrial cells or spheroids. Another option for investigating cytotoxicity of different compounds in spheroid cultures is studying the morphological integrity after the treatment. This was also done in the current study by capturing images of the spheroids every 24 h after treatment with the inverted light microscope.

3.4.9 Invasion and proliferation analysis

One of the important similarities cancer and endometriosis possess, that is also one of the key factors for disease progression, is the invasiveness of the diseased cells [133]. Therefore, in Paper II, an assay that evaluates the cells' capability of invading through the extracellular matrix resembling Matrigel was conducted. 48 h after transfection with therapeutic CPP/siRNA NPs, the cells were detached and transferred into Matrigel-coated inserts of the invasion chambers. FBS-containing media was used as a chemoattractant and 24 h later the invaded cells were fixed with methanol and stained with methylene blue and eosin. Cells were counted under an inverted light microscope. To add, it has previously been shown for endometriosis, that a synthetic steroid danazol inhibits the proliferation of endometriotic cells in vitro in a dose-dependent manner [134]. In Paper II, it was tested, if it is possible to increase endometriotic cell therapeutic response to different danazol concentrations by combining it with CPP/siRNA NP treatment. For that, the endometriotic cells were supplied with NPs and right after with danazol. As a first assay, proliferation rate was measured 48 h post-treatment. The cells were fixed with methanol and stained with methylene blue, which absorbance was measured after elution with HCl. To further assess the physiological significance of NP–danazol co-treatment, endometriotic cell invasion capability through the Matrigel was measured as described above.

3.4.10 Nanoparticle 3D tissue penetration

To study the nanoparticles' uptake in 3D cultures and the potential of tissue penetration, the spheroids were treated with fluorescently labelled NPs in Paper II and III. In Paper II, they were live-imaged under confocal microscope at certain timepoints. In Paper III, the spheroids were fixed and thereafter the nuclei were stained with DAPI. The images were acquired using a confocal laser scanning microscope.

3.4.11 Cell migration assessment from 3D spheroids

One of the common crucial steps in the progression of tumors or endometriotic lesions, is the cells' migrative capability. Therefore, in Paper II we adopted a spheroid-based functional assay from previously described protocols [135,136] to investigate the effect of downregulating therapeutic genes RRM2 or VEGF. The assay was performed using a representative highly migrative tumor spheroid model. 48 h after transfection, the spheroids were planted on gelatin-coated surface and tumor cells were given 3 days to migrate over the surface. Then, the cultures were fixed with PFA and stained with methylene blue and eosin and photographed under stereomicroscope. The cell migration surface area was measured using ImageJ software.

3.5 Immunostaining of clinical tissue samples

To evaluate the translational potential of PL1-mediated targeting in Paper III, the AgNP binding was tested ex vivo on clinical eutopic endometrial and peritoneal endometriosis tissue samples. In addition, the expression of PL1 receptors and the histological localization of stromal and endothelial cells on the tissue cryosections was captured. For immunofluorescence (IF) staining and AgNP binding experiments, sections were fixed in methanol and permeabilized with Triton-X. For NP binding, CF555-labeled AgNPs were applied on sections at RT for 2 h. For IF, primary antibody was incubated in blocking buffer that contained the serum of host animal of the secondary antibody. All sections were thereafter stained with DAPI and visualized under confocal microscope. The tissue sections that underwent IHC, where fixed in PFA, followed by primary antibody incubation plus DAB-staining and visualized under tissue slide scanner.

3.6 In vivo therapeutic gene knockdown

The final aim of Paper I was to test the in vivo efficacy of the NPs formed using the most effective peptide NF70. The blood coagulation factor VII was chosen as a proof-of-concept target for therapeutic siRNA delivery. The CPP/siVII NPs were formed at MR30, whereas 1.6 mg/kg siRNA dose was used per one BALB/c mouse. As negative controls, untreated mice and pure siVII injection was used. As a positive control for siRNA delivery and in vivo efficacy, the commercially available transfection reagent in vivo-jetPEI® was used. Another positive control was the hydrodynamic injection of the siVII. The hydrodynamic injection method is an effective procedure for delivering nucleic acids into the liver of the recipient animal by rapid intravenous (i.v.) injection of a large volume of the nucleic acid solution. Nevertheless, this method is not clinically applicable on humans, it can only be used as a technical control in preclinical animal experiments [137]. Glucose was added to the formed NPs or controls to achieve isotonic solution that was directly injected i.v. via tail vein. Since factor VII is mainly synthesized in the liver cells, the liver tissues were harvested and homogenized with the TRIzol® reagent 48 h after treatment. Total mRNA was isolated and cDNA was subsequentially synthesized. Thereafter the gene expression levels were measured using qRT-PCR.

4. RESULTS AND DISCUSSION

4.1 Enhancement of siRNA transfection by the optimization of fatty acid length and histidine content in the CPP (Paper I)

In this study, a new histidine-containing series of NickFect peptides that could efficiently transport siRNA into cells both in vitro and in vivo was being rationally designed. For that, the net charge, hydrophobicity, pH-sensitivity and charge distribution of the NF55 peptide was modified and optimized. We have previously demonstrated the importance of positive net charge and the fatty acid tail of Transportan analogs in non-covalent NP formation and stabilization [138]. Therefore, to start with, two amino acid replacements were made in the sequence, which added one positive charge and increased the hydrophobicity of the original NF55 peptide. This resulted in the positively charged C-terminal part with cationic residues aligned on one side of a theoretical α-helix. Histidine residues have neutral charge and are hydrophobic in physiologic neutral pH environment, but in acidic conditions they become hydrophilic and cationic due to protonation. This induces osmotic swelling of the endosome and leads to endosomal escape. The protonation also induces the conformational change of the peptides to α-helical structures that promote the endosomal escape by interacting with the endosomal membrane [65]. Hence, different numbers of histidine residues were included in the CPP sequence to increase the oligonucleotide delivery efficacy. The optimization of the hydrophobicity by the length of the fatty acid tail has proven to play a role in CPP/ON transfection efficacy [139]. Thus, to determine the optimal length for each peptide sequence, saturated fatty acid moieties with the carbon number of C18, C20 or C22 were tested.

All synthesized histidine-rich NF55 analogs (Table 1) were first screened for transfection efficacy using luciferase reporter gene knockdown experiments. The best-performing CPPs in facilitating siLuc delivery at different peptide to siRNA molar ratios (MR) were chosen for further experiments. At MR30, PF6, NF71 (H4-C18) and NF70 (H6-C20), were equally efficient and at MR40, NF70 was slightly more effective in downregulating luciferase. When analyzing the signal of another reporter gene, GFP, after CPP/siGFP treatment, the confocal microscopy images revealed that the transfection efficacy was the highest again in the case of siGFP NPs formed with PF6, NF71 and NF70. The flow cytometry analysis of GFP-positive cells after CPP/siGFP treatment showed that the lowest number of GFP-positive cells was in the PF6/siGFP group. This was followed by slightly less efficient transfection mediated by NF55, NF71 and NF70 treatment.

The best-performing histidine-rich CPPs throughout the siRNA transfection experiments were NF70 and NF71, whereas the latter being the most efficient. On the other hand, the cellular toxicity was higher in case of NF71, whereas NF70, while still being extremely efficient, exhibited no toxic effects even at high concentrations. All peptides that had previously facilitated high gene knockdown,

encapsulated siRNA to regular, homogeneous and mostly spherical NPs, detected by TEM.

There is an optimal range of stability that the nanoparticle has to fall into. The NPs have to be stable enough to resist the primary degradative or neutralizing blood components in order to achieve desired functional changes [124]. On the other hand, in order to facilitate efficient transfection, the cargo has to be released when reaching the target site. Therefore, the stability of the NPs was evaluated using heparin displacement assay that mirrors the condensation strength of the CPP/siRNA particles and a Proteinase K assay to evaluate the nanoparticles' resistance against enzymatic degradation. As a result, it become evident that the addition of histidines to NF55 backbone and increasing the fatty acid length increased the stability of the CPP/siRNA complexes against proteolytic degradation and their packing ability. NPs formed with C20 fatty acid peptide may be too stable for releasing the siRNA, as indicated by the lower transfection efficacy. NF70/siRNA nanoparticles have a stability profile between that of the H4-C18/siRNA and H8-C20/siRNA nanoparticles, and the highest gene knockdown. Hence, NF70 probably has an optimal stability profile, as the peptides with higher stability show lower transfection efficacy.

Interactions with the cell membrane are vital for the nanoparticles' internalization into cells. Regardless, high membrane activity may be toxic to cells. Red blood cells have been used for assessing the ex vivo membrane activity and the endosomal escape of pH-sensitive delivery agents [140]. In this study, NF70 had the most advantageous profile: a low membrane activity at physiologic pH that increased in the low-pH environment. The addition of siRNA to the peptide solution and formation of the respective complexes decreased the hemolytic activity approximately two-fold, suggesting that the membrane activity could be mediated by the free or accessible CPP that has not been neutralized by the interaction with the negatively charged siRNA.

Finally, to test the in vivo efficacy of histidine-rich peptides, a blood coagulation factor VII was chosen as siRNA target. This vitamin K dependent enzyme belongs to the blood coagulation cascade and is mainly synthesized in the liver cells. siFVII NPs formulated using the most promising CPP in vitro, NF70, were also successful in mediating siRNA-induced knockdown of factor VII in mouse liver. A single intravenous injection resulted in an almost 60% knockdown of the target gene.

To summarize Paper I, we have developed a new CPP with a completely redesigned N-terminus that displays pH-sensitive response to surrounding environment. This peptide, NF70, is highly successful in siRNA condensation, cargo protection against degradation and mediating target gene knockdown both in mammalian cell culture and in a mouse in vivo model (Figure 11).

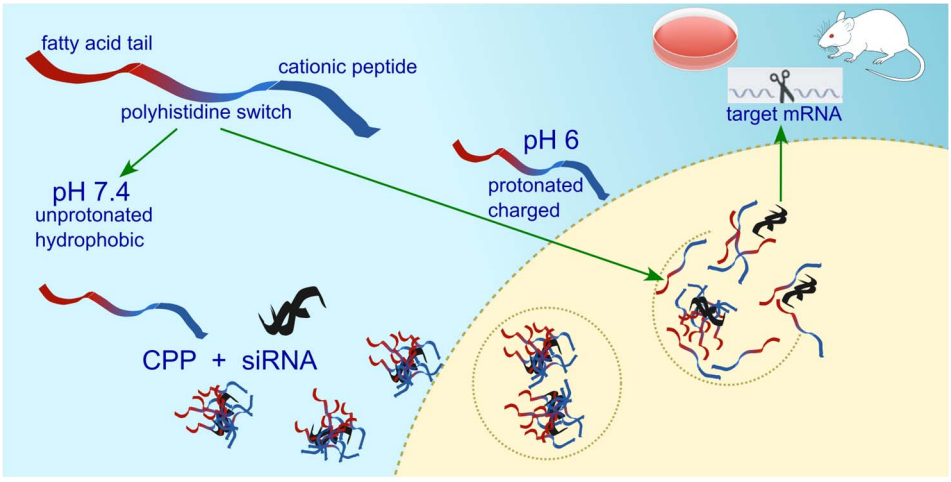

Figure 11. Histidine-rich CPPs become protonated in lysosomes and mediate efficient siRNA delivery and RNA interference in vitro and in vivo. Some details used are modified from Wikimedia Commons [141,142].

4.2 Cell-Penetrating Peptide and siRNA-Mediated Therapeutic Effects on Endometriosis and Cancer In Vitro Models (Paper II)

To further investigate the potential of NF70 as a drug delivery vector, the next step was to test its efficacy in parallel with the most successful siRNA delivery vector so far, PF6, in different disease models of endometriosis and cancer. Despite marked differences, both of these conditions still exhibit similarities, like adhesive, proliferative, migrative and invasive properties. Thus, in the current study, the therapeutic effects of CPP/siRNA NPs targeting known genes in anti-cancer therapy, RRM2 and VEGF, were investigated using various in vitro models. As a first step, the siRNAs were validated, transfection conditions optimized and resulting changes in tumor cells' gene expression evaluated. As expected, CPP NPs with siRRM2 or siVEGF proved to induce significant target gene knock-down. RRM2 downregulation was accompanied by RRM2 protein level decrease in tumor cells after PF6/siRRM2 treatment. Since RR is a crucial enzyme for DNA synthesis and cell proliferation, its subunit RRM2 downregulation induces cell cycle arrest in G1/S phase. We performed the flow cytometry analysis, which confirmed the successful cell cycle arrest after CPP/siRRM2 treatment.

Relying on the similarities described for cancer and endometriosis [133,143], as a next step, we aimed to investigate the effect of the siRRM2 and siVEGF NP formulations on endometriotic cells. In terms of histology and cellular composition, endometriotic lesions often have both endometrial components: epithelial glands and stromal cells surrounding the glands. In some cases though, it has been shown that only stromal cells are present in the lesion [12]. Therefore, as a proof

of concept, primary stromal cells isolated from patient samples of peritoneal and ovarian endometriosis, were used. Endometriotic cells' RRM2 and VEGF gene expression analysis after CPP/siRNA treatment revealed that PF6 and NF70 NPs are both capable of inducing significant target gene knockdown in the case of both endometriosis subtypes.

Since we suggested RRM2 as a novel target for endometriosis therapy, we aimed to further investigate the effect of its downregulation to the cells. For that, the determination of RRM2 protein levels after transfection showed both CPP/siRNA nanoparticles being capable of inducing RRM2 protein decrease in peritoneal and ovarian endometriotic cells. In addition, the cell cycle analysis of endometriotic primary cells after transfection with CPP/siRRM2 NPs revealed that cell cycle arrest in G1/S phase similar to that seen in tumor cells was induced.

To better mirror the natural physiological environment of the pathological tissues, the addition of another component, extracellular matrix, was investigated. This provides the possibility of measuring the invasive capability of the diseased cells, that has shown to play an important role in the progression of both, cancer and endometriosis [133]. We evaluated the cultured cells' invasive capability through the Matrigel extracellular matrix due to chemoattraction to serum components after applying CPP/siRNA NPs. A significant decrease of up to 97% in tumor cell invasiveness was observed after CPP-mediated delivery of siRRM2 and VEGF. In the case of primary peritoneal and ovarian endometriotic cells, the invasive capability was also significantly hindered up to 80%.

Regular monolayer cell cultures are still artificial and lack several characteristics of the live tissue, where the cells exist within a dynamic three-dimensional (3D) microenvironment. Furthermore, 3D cultures have shown to be more drug resistant and also, they enable the evaluation of a major challenge in drug development – the tissue penetration ability. Thus, the 3D cell cultures can be considered as an intermediate step between regular monolayer cells and in vivo experiments, which enables the reduction of experimental animals used in the drug development research [29]. Therefore, advanced, multilayered 3D cellular models were used in this work for disease modelling. To start, the tissue penetration of fluorescently labelled CPP/siRNA NPs was evaluated in tumor and peritoneal endometriotic primary spheroids. During confocal imaging, the NP signal was mainly seen in the cells situated in the outer rim of the spheroids. After confirmation of successful spheroid transfection, the spheroids were treated with therapeutic CPP/siRNA NPs and the significant downregulation of RRM2 and VEGF in 3D cultures was confirmed.

PF6 had been extensively studied previously, but NF70 was a novel peptide developed in Paper I with most recent findings demonstrating it successfully delivers therapeutic miRNA into primary human keratinocytes and dendritic cells [144]. During further studies on NF70 performance, it exhibited high efficacy in transfecting primary 3D cell cultures. Lipofectamine RNAiMAX, on the other hand, did not perform as invariably during experiments with 3D cultures, even if being used at equivalent siRNA concentrations to CPPs.

For disease-specific studies, we adopted a spheroid-based functional assay [135,136] to investigate the RRM2 and VEGF downregulation impact on cell migration – another important factor for tumor progression. The measurement of the surface area covered by the migrated tumor cells from the spheroid demonstrated significant inhibition in all of the therapeutic CPP/siRNA treatment groups.

For endometriosis, we hypothesized, that it might be possible to reduce the administered amount of the synthetic steroid danazol by combining it with the NP treatment strategy. For that, the NP–danazol co-treatment effect on endometriotic cell proliferation and invasion was investigated. When combining therapeutic NF70/siRNA NP treatment with different danazol concentrations, there was indeed significant synergistic effect present, best seen when using danazol 20 μM concentration. Only approximately 5% of the cells were capable of maintaining their original invasive capability.

RRM2 is expressed in all cells in the body undergoing mitosis [10], VEGF in all tis-sues forming blood vessels [9], and the side effects of systemic administration of danazol have been well described [5]. We demonstrated a remarkable possibility for reducing danazol administration amount by combining it with therapeutic CPP/siRNA NPs. Nevertheless, we decided that before proceeding with in vivo validation studies, further development of the nanoparticles is necessary in order to avoid the systemic side effects of therapeutic siRNA or danazol. An appealing option for achieving specific endometriosis tissue targeting could be the functionalization of described NPs by homing peptides. This was the main reason for initiating the search for a suitable targeting peptide in Paper III.

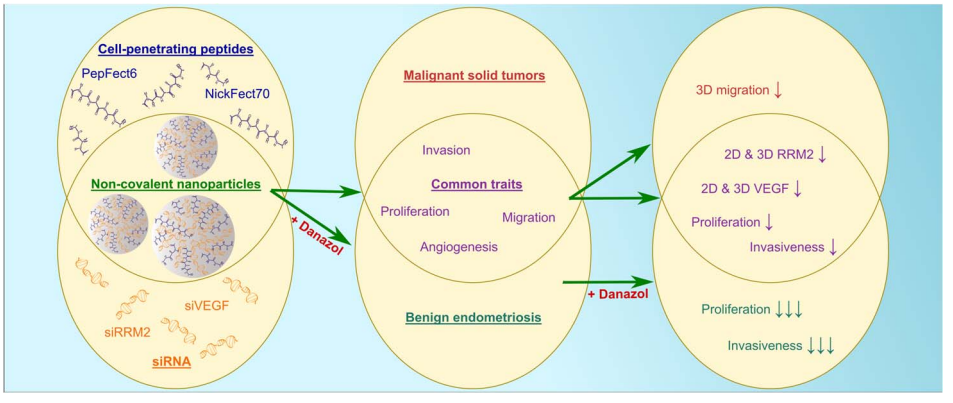

Figure 12. Therapeutic effects of endometriotic and cancer cells in vitro models after various CPP/siRNA treatments.

4.3 Homing Peptide-Based Targeting of Tenascin-C and Fibronectin in Endometriosis (Paper III)

Targeting of tissues of interest with homing peptides may help to overcome the issue of unspecific accumulation of therapeutics in healthy organs. In the current study, the aim was to identify a homing peptide specifically targeting endometriosis. To start with, we tested a panel of peptide phages for the binding to common endometriosis-modelling cell-lines: eutopic stromal HESC and endometriosis epithelial 12Z cells. Based on the described common traits of cancer and endometriosis [133,143] and the results of Paper II, we postulated that some tumor homing peptides could also be suitable for the targeting of endometriosis. As a result, PL1 peptide targeting TNC-C and Fn-EDB exhibited the strongest binding to 12Z and HESC cells. Phages displaying another TNC-C targeting peptide, PL3 and also a known tumor homing peptide iRGD, showed significant binding to endometriotic cells. At the same time, the two previously suggested endometriosis homing peptides (in this work referred to as EM1 [119] and EM2 [120]) were not successful in our binding experiments to 12Z and HESC cells. The receptor of EM1 peptide (z13) was identified to be the cyclic nucleotide-gated channel β3 (CNGB3), a sorting pathway protein highly expressed in endometrial glandular epithelial cells and peritoneal surfaces in clinical endometriosis samples. EM1 peptide was identified by intermittent negative in vivo phage display in mouse peritoneal cavity combined with regular phage binding to Ishikawa cells [119]. These are endometrial adenocarcinoma cells by nature, which means z13 was identified using cancer cells, which might be the reason why our in vitro binding results from endometriosis and eutopic endometrial cells do not correlate with the z13 peptide original results. EM2 on the other hand was identified by phage biopanning on clinical endometriosis samples [120] and was shown to bind to ectopic endometrial stromal cells. Our results confirm the binding of EM2 to HESC cells, although remaining to modest binding levels compared to PL1 peptide.

Therefore, in this study we concentrated on investigating the proof-of-concept theranostic potential of co-targeting PL1 receptors, TNC-C and Fn-EDB, in endometriosis. For that, the expression of the homing peptides' receptors was evaluated by ICC and visualized under confocal microscope. As an outcome, TNC-C, Fn-EDB, αv integrins, and a lesser amount of NRP-1 were expressed by 12Z and HESC cells. The internalization of fluorescent peptide-functionalized AgNPs was measured by flow cytometry after removing the non-internalized particles using the etching solution. The results clearly show that the PL1-AgNPs bind and internalize into both cell lines. Next, the cellular internalization and penetration of fluorescently labeled PL1-AgNPs into 12Z spheroids was studied by confocal fluorescence microscopy. At the beginning, the PL1-AgNPs internalized in the spheroids' outer rim and after 20 h of incubation, the AgNPs were also visualized deeper in the spheroids, whereas non-targeted biotin-AgNPs did not bind to the spheroids.

To continue, the cytotoxicity of endometriotic cells and spheroids was evaluated after the treatment with PL1-AgNPs loaded with an anti-mitotic agent MMAE. For 2D, at the highest MMAE concentration (30 nM), the treatment resulted in a ~50% decrease in the number of viable 12Z cells and in a ~80% decrease of viable HESC cells, whereas non-targeted MMAE-AgNPs did not have any cytotoxic effect. The higher cytotoxic effect observed in HESC compared with 12Z cells could be due to the different chemosensitivity of endometriotic epithelial and stromal cells, similar to differential chemoresponsiveness observed between malignant epithelial and stromal cells of malignant carcinomas [145]. Also for 3D 12Z cultures, the cell viability decreased significantly after the treatment with PL1-MMAE-AgNPs. In addition, the damage of the spheroid integrity was microscopically evaluated. It became evident that PL1-MMAE-AgNPs induce blebbing and loss of the compact structure of the 12Z spheroids. These results show that the therapeutic PL1-functionalized NPs are capable of efficiently delivering an intracellularly-acting drug inside the endometriotic 2D and 3D cultures that facilitates cytotoxic effects.

What is more, the signal of PL1-AgNPs was observed in the cryosections of clinical endometriosis lesions in areas close to the blood vessels and positive for the receptors, TNC-C and Fn-EDB, while no signal was seen in normal endometrium. TNC-C has also previously been shown to be expressed around angiogenic vasculature, proliferating cells and tumor stroma with a predominantly perivascular staining pattern [146]. When considering the histological characteristics of endometriosis lesions, it is important to note that the proportion of epithelial glands surrounded by the stromal cells forms only a part of the actual size of the lesion. This is due to endometriosis being considered a neuro-inflammatory angiogenesis-dependent disease, where the nerves and newly formed blood vessels surround the endometriotic lesions [37]. To add, the laparoscopic resection of the lesions often results in the recurrence of the disease. One reason for this is thought to be that during the surgery, insufficient amount of tissue is resected, since it is only possible to determine the size of the lesion by visual assessment of the surgeon. Therefore, the fact that we are capable of marking a wider area co-localizing with blood vessels, that surrounds the endometriotic cells, adds to the theranostic potential of PL1-guided nanoparticles.

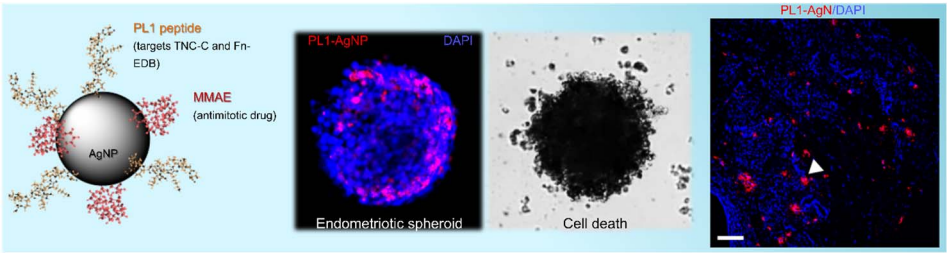

Figure 13. PL1-functionalized AgNPs penetrate 3D endometriotic spheroids, induce spheroid disintegration when loaded with MMAE and bind to endometriosis tissue sections ex vivo.

CONCLUSIONS

The key findings from the current thesis are as follows:

Paper I In this study, a new histidine-rich series of NickFect peptides based on the sequence of NF55, that could efficiently transport siRNA into cells both in vitro and in vivo was rationally designed. For that, the net charge, hydrophobicity, pH-sensitivity and charge distribution of the peptide was modified and optimized. We demonstrated the significance of number of histidines included in a peptide sequence and their position and distribution in the designed CPPs. In addition, we showed the importance of a fatty acid length modification to the characteristics of the assembled CPP/siRNA NPs. The most effective CPP designed, a pH-sensitive NF70, is capable of facilitating efficient cargo delivery and improved endosomal escape in vivo and in vitro in a non-toxic manner.

Paper II The current study revealed that PF6 and NF70 are both highly successful in transfecting primary cells and 3D spheroids. This leads to induction of relevant siRNA-induced phenotypic changes like in vitro inhibition of invasion and migration. We also demonstrated that therapeutic CPP/siRNA NP and danazol co-treatment synergistically reduces endometriotic cell proliferation and invasion. According to our knowledge, this study is the first one aiming to develop a synthetic CPP-based delivery approach for endometriosis treatment. In addition, we suggested a novel target for endometriosis therapy, ribonucleotide reductase. As a proof-of-principle, we proposed a CPP and siRNA-mediated therapeutic strategy for cancer and endometriosis, that we aimed to further improve for translational purposes.

Paper III The aim of the study was to identify a homing peptide that would specifically target endometriosis. The peptide binding studies to endometriotic cells revealed PL1 as the most promising homing peptide. The PL1-functionalization of AgNPs resulted in efficient internalization in endometriotic 2D and 3D cultures and potentiating the cytotoxic effects of AgNP-loaded intracellularly-acting drug MMAE. Furthermore, we observed PL1-AgNP accumulation in the areas of cryosections from human endometriosis samples that were positive for TNC-C and Fn-EDB expression, whereas the sections of normal endometrium remained negative, suggesting translational relevance of PL1-based targeting strategies. Our findings encourage further studies on PL1-based targeting of therapeutic and imaging agents to endometriosis lesions.

SUMMARY IN ESTONIAN

Endometrioosi raviks sobilikud peptiidsed ravimikandjad ja prekliinilised nanomeditsiini rakendused

Endometrioos on sage günekoloogiline haigus, mis esineb kuni 10% viljakas eas naistest. Haigust iseloomustavad väljaspool emakat kasvavad endomeetriumi kolded, mis tekitavad patsientidel kroonilist kõhuvalu ja sageli ka viljatust. Kasutusel olevad endometrioosi ravi- ja diagnostikavõimalused on piiratud. Tihti ei oma farmakoloogiline ravi pikaajalist efekti ning süsteemse manustamise tõttu põhjustab see kõrvaltoimeid. Seetõttu on vajalik uute endometrioosi ravistrateegiate väljatöötamine. Paljud potentsiaalsed ravi-sihtmärgid asuvad aga tegelikkuses haiguskoldes rakkude sees. Seetõttu on mitmete haiguste puhul suurimaks väljakutseks see, et ravimid peavad kohale jõudma oma rakusiseste sihtmärkmolekulideni. Sellest tulenevalt oli antud töö eesmärgiks välja töötada või uuskasutada peptiididel põhinevaid ravimkandjaid nanomeditsiini rakenduste jaoks, keskendudes põhiliselt endometrioosile.

Alustuseks kasutati geenikspressiooni reguleerivate oligonukleotiidide transpordiks rakku sisenevaid peptiide ehk süstikpeptiide. Esmalt disainiti seeria uusi histidiine sisaldavaid peptiide, mis reageerivad keskkonna füüsilistele parameetritele. Kõige edukam peptiid, pH-tundlik NickFect70, suutis efektiivselt lühikest interfereeruvat RNAd (siRNA) rakkudesse sisse viia nii in vitro kui ka in vivo tingimustes. Teiseks testiti süstikpeptiid/siRNA nanopartiklite ravimpotentsiaali vähi ja endometrioosi ühekihilistes ja 3D sferoidi rakukultuuri mudelites. Selleks kasutati kahe siRNA efektiivsuse mõõtmist, mille sihtmärkgeenid mõjutavad haiguskudede kasvu ja progresseerumist. Tulemusena vähenesid vähi ja endometrioosi eelkliiniliste mudelite rakkude proliferatsioon, invasioon ja migratsioon. Lisaks tekitas geeniteraapia ja hormoonravi kombineerimine sünergilise endometrioosirakkude proliferatsiooni ja invasiooni pidurdumise. Olenemata süstikpeptiidide kõrgest rakku sisenemise efektiivsusest ei oma nad koespetsiifilisust ja see võib süsteemse in vivo manustamise korral viia ravimite ebaspetsiifilise tervetesse kudedesse akumuleerumiseni. Seetõttu uuriti viimase sammuna sihtmärk-kude spetsiifiliselt äratundvate peptiidide ehk kullerpeptiidide kasutusvõimalusi. Kõige edukamaks peptiidiks endometrioosi puhul osutus PL1 kullerpeptiid, mis on varasemalt näidanud selektiivset seondumist paiksete kasvajate rakuvälise maatriksi komponentidele veresoonte ümbruses, täpsemalt fibronektiini ja tenastsiin-C konkreetsetele isovormidele. Sünteetilise PL1 peptiidiga kaetud hõbe-nanopartiklid näitasid spetsiifilist kuhjumist 2D ja 3D rakukultuurides ja seondumist kliinilistele endometrioosi koeproovidele.

Kokkuvõtvalt näitasid antud doktoritöö tulemused süstikpeptiid/siRNA nanopartiklite abil kahe terapeutilise geeni mahasurumise sobivust endometrioosi raviks. Kombineerides nanopartikleid hormonaalse ravimiga, suurenes raviefekt veelgi. Lisaks eelnevale näitasid PL1-kaetud nanopartiklid suurt potentsiaali endometrioosi koespetsiifiliste ravimite ja diagnostikameetodite väljatöötamiseks. Kõike arvesse võttes pakuvad käesoleva doktoritöö tulemused välja mitu siirdemeditsiinilise väärtusega peptiididel põhinevat nanomeditsiini rakendust endometrioosi raviks.

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Publications

Simón-Gracia L*, **Kiisholts K***, Petrikaitė V, Tobi A, Saare M, Lingasamy P, Peters M, Salumets A, Teesalu T (2021) Homing Peptide-Based Targeting of Tenascin-C and Fibronectin in Endometriosis. Nanomaterials 11:3257
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Publikatsioonid

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