Review article

siRNA in ovarian cancer – Delivery strategies and targets for therapy

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ABSTRACT

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy, and the sixth leading cause of cancer related death in women overall. Despite improved surgical techniques and advances in chemotherapy, mortality hardly decreased over the last twenty years. The major problem is that (micro)metastases persevere in the abdominal cavity, causing incurable tumor recurrence. Therefore, there is an imminent need for new therapeutic strategies.

Oligonucleotide (ON) based therapies such as RNA interference (RNAi) provide the possibility to specifically address disease-related pathways. However, small interfering RNA (siRNA) molecules are unable to enter cells without a drug delivery system. Therefore, nanocarriers have been developed to aid intracellular delivery of siRNA. EOC is, in most cases, confined to the abdominal cavity, providing the possibility for peritoneal drug delivery. As a consequence, EOC should be an ideal candidate for ON therapies as intraperitoneal delivery reduces sequestration of drug formulations in other organs.

In this review, we will discuss delivery strategies and siRNA targets that have been tested in EOC. Delivery strategies cover the full range of delivery approaches from polymers to exotic delivery strategies like microbubble based nanoparticles. For siRNA targets, those that aim at re-sensitizing the tumor cells to chemotherapy can be discriminated from those that reduce growth and metastasis of the tumor cells.

Despite preclinical successes and the advantage that intraperitoneal delivery holds over systemic delivery, no strategy has made it into the clinic yet. We postulate that confirmatory studies that combine the most promising delivery approaches with the most promising targets are required to reach a consensus on those formulations that should be pursued for further (pre-)clinical research.

1. Introduction

Despite its relatively low incidence, epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy in developed countries, and the sixth leading cause of cancer-related deaths among women [1]. The mean age of onset is 63 and 5 year survival is only 46.5% of all stages and subtypes combined, with the epithelial type as the most common and most lethal subtype [2]. Survival is particularly low due to the late onset of symptoms and diagnosis in an advanced stage. Advanced stage EOC treatment consists of (neo)adjuvant chemotherapy and cytoreductive surgery [3]. The aim of the procedure is to remove as much tumor as possible. However, (micro)metastases almost always persevere within the abdominal cavity, causing tumor recurrence. The median progression free survival of advanced ovarian cancer is approximately 18 months and a recurring tumor is typically characterized by increased resistance to chemotherapeutics [3]. Curation is unfortunately not possible with current treatment modalities. Therefore, novel therapeutic options are necessary.

By altering gene expression, it could be possible to decrease tumor progression and/or increase sensitivity to cytotoxic agents. RNA interference (RNAi) is a promising method used for this purpose [4]. RNAi can be induced in several ways and small interfering RNA (siRNA) molecules are well-known class of double stranded oligonucleotides of 20–25 base pairs that specifically induce degradation of mRNA, thereby creating efficient gene knockdown [4].

The main advantages of siRNA are high specificity, high efficiency, and low toxicity [5,6]. However, naked siRNA molecules are prone to degradation by RNase. Moreover, siRNA and other oligonucleotides are unable to permeate the plasma membrane and therefore do not reach sufficient intracellular concentrations to induce effective gene knockdown. Safe
and efficient transfection methods have therefore been developed. For clinical applications, these drug delivery systems mostly comprise non-viral nanoparticles [7].

Confinement in the intraperitoneal cavity, also at advanced stage, is a key characteristic of EOC. A recent review states that FIGO (International Federation of Gynaecology and Obstetrics) stage IV disease, which refers to the stage of disease with extraperitoneal involvement, is present in only 12–33% of the cases at initial diagnose [8]. With an incidence of 33–53%, pleural effusion is the most common extraperitoneal manifestation of EOC, followed by liver, subcutaneous/abdominal wall, and extra-abdominal lymph nodes, whereas brain and bone metastases are uncommon [8]. Therefore, local treatment of the metastatic tumor with IP chemotherapy after cytoreductive surgery is a feasible method that increases survival in ovarian cancer patients [9]. Also for nanoparticles, IP application imposes very different boundary conditions on biodistribution.

This review will provide an overview of delivery systems and their respective targets for siRNA delivery in the context of ovarian cancer. The Pubmed database was searched according to the search strategy shown in the supplementary material S1 and key features of the literature are summarized in comprehensive Tables 1–6 and in graphical form in Figs. 1 and 3.

The most widely used nanoparticle formulations are polymer and lipid based (Fig. 1a). Polyethyleneimine (PEI) and chitosan are the most commonly used polymers. Polymer based nanocarriers are often positively charged and are therefore able to bind oligonucleotides via electrostatic interactions with the anionic backbone of nucleic acids. The positive charge of these polymers also enables them to enter cells more efficiently than naked siRNA.

Lipid based nanoparticles comprise liposomes and lipid nanoparticles (LNPs, Fig. 1b). Liposomes consist of a lipid bilayer with an aqueous core into which siRNA can be loaded. This is in contrast to LNPs, which are less structured aggregates of lipid micelles and oligonucleotides. Again, the presence of cationic and protonatable groups is crucial for mediating the interaction with the plasma membrane and delivery of encapsulated oligonucleotides. In addition, strategies such as dendrimers, mesoporous silica nanoparticles, and microbubbles will be discussed (Fig. 1c–e).

Although the molecular make up of nanoparticles can vary greatly, they are all characterized by the capacity for further functionalization. These carriers of increasing complexity aim at enhancing cell type selectivity, uptake efficiency, anticancer activity and circulation time [6]. Polyethylene glycol (PEG) is the most frequently used shielding agent to increase circulation time and avoid recognition by the immune system [10]. Targeting moieties include for example antibodies or small molecules such as folate. Cellular uptake efficiency can furthermore be enhanced by using cell-penetrating peptides [11–13].

Unfortunately, there has still been no translation of siRNA-mediated gene targeting into the clinic despite extensive preclinical research and the urgent medical need. We identify several potential drawbacks of the current state-of-the-art and try to explain why these inhibit further development into a clinically applicable therapy. We argue that the sheer abundance of delivery strategies and the lack of cross validation between these methods stalls further development of siRNA delivery systems in ovarian cancer.

There seems to be a tendency towards increasingly complex delivery systems instead of identifying the minimal platform for reaching sufficient activity. The same holds for the choice of the siRNA target. A number of genes has been targeted for knockdown, but no research is devoted towards reaching a consensus as to which target would eventually have the strongest potential in the clinic.

2. Polymer based nanoparticles

2.1. Polyethyleneimine (PEI) based nanoparticles

Polyethyleneimine (PEI) based nanoparticles have been widely used in the clinic despite extensive preclinical research and the urgent medical need. We identify several potential drawbacks of the current state-of-the-art and try to explain why these inhibit further development into a clinically applicable therapy. We argue that the sheer abundance of delivery strategies and the lack of cross validation between these methods stalls further development of siRNA delivery systems in ovarian cancer.

There seems to be a tendency towards increasingly complex delivery systems instead of identifying the minimal platform for reaching sufficient activity. The same holds for the choice of the siRNA target. A number of genes has been targeted for knockdown, but no research is devoted towards reaching a consensus as to which target would eventually have the strongest potential in the clinic.

Table 1

<table>
<thead>
<tr>
<th>Characteristics of polyethyleneimine (PEI) based nanoparticles.</th>
<th>Article</th>
<th>NP composition</th>
<th>Size (nm)</th>
<th>Zeta potential (mV)</th>
<th>siRNA target</th>
<th>Chemotherapeutic</th>
<th>Cell line in vivo</th>
<th>Mouse model</th>
<th>Drug administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zou [47]</td>
<td>FA-PEG-PEI-PCL</td>
<td>188 to 191</td>
<td>$^{+}5.32$ to $^{+}6.51$</td>
<td>PTX</td>
<td>OVCAR-8TR</td>
<td>Female nude BALB/c</td>
<td>SC</td>
<td>IV</td>
<td>IP PTX</td>
</tr>
<tr>
<td>Li J [22]</td>
<td>HER2-PEG-PLL-AED</td>
<td>72 to 3.1</td>
<td>$^{+}5.81$</td>
<td>Bcl-2</td>
<td>Dox in NP</td>
<td>SK-OV-3</td>
<td>Female nude BALB/c</td>
<td>SC</td>
<td>IV</td>
</tr>
<tr>
<td>Yang [27]</td>
<td>HA-PEG/HA-PEI</td>
<td>173.3 ± 4.4</td>
<td>HA-PEG/HA-PEI</td>
<td>MDR-1</td>
<td>PTX</td>
<td>SK-OV-3</td>
<td>&amp; SK-OV-3</td>
<td>SC</td>
<td>IV</td>
</tr>
<tr>
<td>Jone [43]</td>
<td>PEM-PEG-FA</td>
<td>167 ± 2</td>
<td>$^{+}4.8$</td>
<td>CD44</td>
<td>MDR1</td>
<td>SK-OV-3</td>
<td>&amp; SK-OV-3</td>
<td>SC</td>
<td>IV</td>
</tr>
<tr>
<td>Zou [17]</td>
<td>HER2-PEG-PLL-AED</td>
<td>198 to 191</td>
<td>$^{+}5.32$ to $^{+}6.51$</td>
<td>PTX</td>
<td>OVCAR-8TR</td>
<td>Female nude BALB/c</td>
<td>SC</td>
<td>IV</td>
<td>IP PTX</td>
</tr>
<tr>
<td>Chen [52]</td>
<td>PEG-PAsp(AED)</td>
<td>72 to 3.1</td>
<td>$^{+}5.81$</td>
<td>XIAP</td>
<td>Bcl-2</td>
<td>Dox in NP</td>
<td>SK-OV-3</td>
<td>Female nude BALB/c</td>
<td>SC</td>
</tr>
<tr>
<td>Teo [21]</td>
<td>PEI-PEG-FA</td>
<td>167 ± 2</td>
<td>$^{+}4.8$</td>
<td>CD44</td>
<td>MDR1</td>
<td>SK-OV-3</td>
<td>&amp; SK-OV-3</td>
<td>SC</td>
<td>IV</td>
</tr>
<tr>
<td>Yang [27]</td>
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<td>HA-PEG/HA-PEI</td>
<td>MDR-1</td>
<td>PTX</td>
<td>SK-OV-3</td>
<td>&amp; SK-OV-3</td>
<td>SC</td>
<td>IV</td>
</tr>
<tr>
<td>Yang [27]</td>
<td>HA-PEG/HA-PEI</td>
<td>173.3 ± 4.4</td>
<td>HA-PEG/HA-PEI</td>
<td>MDR-1</td>
<td>PTX</td>
<td>SK-OV-3</td>
<td>&amp; SK-OV-3</td>
<td>SC</td>
<td>IV</td>
</tr>
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<td>PTX</td>
<td>SK-OV-3</td>
<td>&amp; SK-OV-3</td>
<td>SC</td>
<td>IV</td>
</tr>
<tr>
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<td>HA-PEG/HA-PEI</td>
<td>173.3 ± 4.4</td>
<td>HA-PEG/HA-PEI</td>
<td>MDR-1</td>
<td>PTX</td>
<td>SK-OV-3</td>
<td>&amp; SK-OV-3</td>
<td>SC</td>
<td>IV</td>
</tr>
<tr>
<td>Yang [27]</td>
<td>HA-PEG/HA-PEI</td>
<td>173.3 ± 4.4</td>
<td>HA-PEG/HA-PEI</td>
<td>MDR-1</td>
<td>PTX</td>
<td>SK-OV-3</td>
<td>&amp; SK-OV-3</td>
<td>SC</td>
<td>IV</td>
</tr>
</tbody>
</table>

- Molar ratio of polymer nitrogen:siRNA phosphorus.
- Mass ratio polymer/siRNA.
Table 2
Characteristics of polysaccharide based nanoparticles.

<table>
<thead>
<tr>
<th>Article</th>
<th>NP composition</th>
<th>Size (nm)</th>
<th>Zeta potential (mV)</th>
<th>siRNA/chitosan ratio</th>
<th>Targeted receptor</th>
<th>siRNA target</th>
<th>Chemotherapeutic</th>
<th>Cell line in vitro</th>
<th>Tumor site</th>
<th>Drug administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li [23]</td>
<td>FA/PEG-COL</td>
<td>200.3 ± 4.6</td>
<td>8.6 ± 3</td>
<td>8.6:1</td>
<td>FR</td>
<td>α</td>
<td>None</td>
<td>OVK18#2/macrophage RAW 264.7 (mouse)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Gharpure [55]</td>
<td>CS-TPP</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>SK-OV-3, SK-OV-3TR</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Babu [59]</td>
<td>PLA-CS</td>
<td>~350</td>
<td>+20 to +5</td>
<td>~1:53</td>
<td>None</td>
<td>P62</td>
<td>(plasmid for β5)</td>
<td>Cisplatin resistant 2008/C13, cisplatin sensitive 2008 human ovarian cancer cell lines</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Kobayashi [60]</td>
<td>Dextran-PEG/dextran-hexylamine</td>
<td>101 ± 3</td>
<td>−0.22 ± 2.21</td>
<td>N/A</td>
<td>None</td>
<td>MDR1</td>
<td>Docetaxel in similar NPs</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

* Mass ratio.
* Weight ratio chitosan oligosaccharide lactate:TPP.

Table 3
Characteristics of lipid based nanoparticles.

<table>
<thead>
<tr>
<th>Article</th>
<th>NP composition</th>
<th>Size (nm)</th>
<th>Zeta potential (mV)</th>
<th>siRNA encapsulation efficiency (%)</th>
<th>Targeted receptor</th>
<th>siRNA target</th>
<th>Chemotherapeutic</th>
<th>Cell line in vitro</th>
<th>Tumor site</th>
<th>Drug administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zhao [64]</td>
<td>S100, DMAPA-Chems</td>
<td>100–200</td>
<td>−3.1 ± 0.5</td>
<td>N/A</td>
<td>Notch1</td>
<td>N/A</td>
<td>None</td>
<td>OVCAR-3</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>He [68]</td>
<td>NCP-DOPA-DOTAP/cholesterol/DSPE-PEG2k</td>
<td>156.3 ± 6.7</td>
<td>91.2 ± 4.9</td>
<td>None</td>
<td>Survivin, Bcl-2, MDR1</td>
<td>SK-OV-3</td>
<td>ES-2, OVCAR-3, SK-OV-3 &amp; macrophage Raw264.7 (murine)</td>
<td>Female nude BALB/c</td>
<td>SK-OV-3 SC IT</td>
<td>IP</td>
</tr>
<tr>
<td>Goldberg [77]</td>
<td>Lipidoid + Cholesterol + PEG-ceramide</td>
<td>~50</td>
<td>&gt; 95</td>
<td>None</td>
<td>PARP1</td>
<td>2'-OMe-modified</td>
<td>None</td>
<td>mT2K, mT3K-Luc, T22H, BR5FB1, BR5FB1-Luc</td>
<td>Female nude BALB/c and female FVB/NJ</td>
<td>IP</td>
</tr>
<tr>
<td>Shahzad [72]</td>
<td>rHDL</td>
<td>~10</td>
<td>~3.2</td>
<td>N/A</td>
<td>SR-B1</td>
<td>FAK</td>
<td>None</td>
<td>HeyA8, SK-OV-3, HeyA8-MDR (HCT116 - colon)</td>
<td>Female nude BALB/c and BALB/c and BR5FB1</td>
<td>IP</td>
</tr>
<tr>
<td>Matsui [75]</td>
<td>YSK05-MEND (with cholesterol + PEG-DMG)</td>
<td>460</td>
<td>~1.5</td>
<td>N/A</td>
<td>CD45</td>
<td>None</td>
<td>None</td>
<td>ICR mice tissue</td>
<td>ICR mice</td>
<td>IV, IP</td>
</tr>
<tr>
<td>Salzano [70]</td>
<td>PEG2000-PE</td>
<td>25</td>
<td>~50%</td>
<td>None</td>
<td>survivin</td>
<td>None</td>
<td>Paclitaxel in NPs</td>
<td>SK-OV-3 TR SCID female</td>
<td>SK-OV-3 TR SC IV</td>
<td></td>
</tr>
</tbody>
</table>

FA, folate; PE, polyethylene glycol; COL, chitosan oligosaccharide lactate; TPP, tripolyphosphate; PLA, polyactic acid; CS, chitosan; FA, folic acid; α, folate receptor alpha; HIF-1α, Hypoxia-inducible factor 1-alpha; EZH2, Enhancer of zeste homolog 2; P62, polyubiquitin-binding protein P62, sequestosome-1; β5, proteasome subunit beta type-5; MDR1, multidrug resistance gene 1; N/A, not available – information not included in the corresponding article; SC, subcutaneous; IV, intravenous; IP, intraperitoneal.

**Footnotes**
- a Mass ratio.
- b Weight ratio chitosan oligosaccharide lactate:TPP.
for siRNA delivery (Table 1) [14]. PEI is a cationic polymer consisting of a repeating amine and ethyl unit. This polymer can spontaneously form condensed nanoparticles (polyplexes) via electrostatic interaction with the anionic backbone phosphates of siRNA, which vary in size from 100 to 1000 nm, [15]. The ratio between the number of charges in the formulation is known as the amine/phosphate (N/P) ratio. The molar ratio of polymer/siRNA is mostly selected in a way that cationic groups are present in excess (N/P > 1), resulting in net positively charged nanoparticles. Furthermore, due to the complexion, siRNA is protected from enzymatic degradation and cellular uptake by endocytosis is promoted [16].

Endosomal escape is a major obstacle for all nanoparticles that enter cells via endocytosis [17]. siRNA that is captured inside endosomes does not reach its molecular target and will ultimately be degraded. PEI based nanoparticles overcome this problem by inducing a process called the proton sponge effect [18]. The amines of PEI bind protons in the lysosome and the persistent influx of protons and charge-neutralizing chloride ions causes an increase in osmolarity. This leads to water influx and ultimately to rupture of the lysosomal membrane and release of its content into the cytosol [18].

Although PEI shows high transfection efficiency, this non-biodegradable polymer is also cytotoxic at higher concentrations [19]. A correlation of higher transfection efficiency with higher cytotoxicity is a problem of many cationic transfection agents as exemplified by high molecular weight PEI [20]. To reduce toxicity in potential clinical use, PEI is functionalized with other molecules or polymers such as PEG. In addition, longer circulation time, as afforded by PEGylation, can increase nanoparticle accumulation in a tumor through the enhanced permeation and retention (EPR) effect, which means that macro-molecular compounds exit the leaky tumor vasculature and remain trapped in the tumor microenvironment [21–24].

Unfortunately, along with reducing toxicity, PEGylation also decreases transfection efficiency of PEI polyplexes and other nanoparticles [10,25]. Other strategies to decrease cytotoxicity while maintaining transfection efficiency have therefore been developed and include combining PEI and PEG with other molecules. The first option is the addition of hyaluronic acid (HA) to both molecules [26,27]. The conjugation of PEI with HA results in electrostatic neutralization of the nanoparticles due to the negative charge of HA. Also, HA contributes to the formation of a protective hydrophilic surface. This polysaccharide furthermore specifically binds to CD44 and can therefore be used as a targeting agent to increase cell specificity. CD44 is a cell-surface glycoprotein that is, among other functions, involved in cell-cell contacts, cell migration, angiogenesis, and cell survival [28]. The protein is often upregulated in ovarian cancer, although there is still debate on its function and association with prognosis [29–31].

Talekar et al. [26] and Yang et al. [27], used such nanoparticles loaded with siRNA to target expression of the Multi Drug Resistance Gene 1 (MDR1), which codes for P-glycoprotein (P-gp), in combination with paclitaxel treatment. Almost 90% of deaths in advanced stage ovarian cancer is linked to MDR, and the major contributing factor is MDR1 overexpression [32–34]. Furthermore, combined CD44 and MDR1 expression correlates with progressive EOC [35]. Therefore, Yang et al. [27] used an siRNA against MDR1 in combination with paclitaxel in order to downregulate P-gp and thereby increase chemosensitivity [27]. In vitro, a dose dependent downregulation of P-gp expression was shown on mRNA and protein level. Furthermore, paclitaxel sensitivity increased in both, in vitro and in vivo models. However, HA targeting was only assessed in CD44 expressing cells, which means that macro-molecular compounds exit the leaky tumor vasculature and remain trapped in the tumor microenvironment [21–24].

Talekar et al. combined siRNA against MDR1 with a second siRNA against pyruvate kinase isozyme M-2 (PK-M-2) [26]. This enzyme converts phosphoenolpyruvate into ATP and pyruvate in glycolysis. It is expressed in cells with a high rate of nucleic acid synthesis such as tumor cells [36]. By targeting two important cancer-related pathways, the authors showed that the efficacy of paclitaxel against MDR EOC was improved compared to targeting of MDR1 alone [26]. Even though
CD44 was used as a cell specificity target, the authors did not assess CD44 expression on the model SK-OV-3 cells. In vivo, siRNA uptake was highest in the liver, kidney, and spleen. Gene knockdown was only demonstrated in the tumor and not in other organs. Whereas the tumor only showed minor uptake, tumor volume was smaller compared to control tested in the tumor and not in other organs. Whereas the tumor only showed minor uptake tumor volume was smaller compared to control tested in vivo.

Teo et al. combined PEI with PEG and folic acid (FA) [21]. The folate receptor α (FRα) is overexpressed in the majority of EOCs and is therefore widely studied for specific ovarian cancer cell targeting [37]. Furthermore, FA is little immunogenic, inexpensive, and stable under cellular conditions [23,38]. To improve siRNA transfection, a disulfide bond was introduced between PEG-FA and PEI that made the polymers cleavable in the cytosol [21]. Different combinations of PEI based nanoparticles were used: PEI, PEI-FA, PEI-PEG, and PEI-PEG-FA. N/P ratios between 10 and 80 were studied and higher transfection efficiencies were observed with increased N/P ratios. A decrease in protein expression was observed for the PEI and PEI-FA nanoparticles at N/P ratios of 50 and 80. With a downregulation by 45 to 49%, these transfections were more efficient than those of PEI-PEG and PEI-PEG-FA, which showed efficiencies of 35–38% at the same N/P ratio. However, cell viability decreased by 20% at an N/P ratio of 50 and by 40% at an N/P ratio of 80. PEI nanoparticles showed even more toxicity, respectively 40 and 60% [21]. To enhance an immune response against the tumor, the authors used siRNA against PD-L1. The interaction between programmed cell death protein 1 (PD-1) and its ligand PD-L1 accounts for T cell hyporesponsiveness in epithelial ovarian cancers resulting in a suppressed local immune response [39–41]. Therefore, a co-incubation with T4 engineered T cells was performed. These T cells were engineered to co-express the chimeric antigen receptor (CAR, T1E28z), and targeted the extended ErbB family. T4 engineered T cells showed potent cytotoxicity on ovarian cancer cells that had shown upregulated expression of the HER2/neu receptor in an earlier study [42]. Co-incubation of T cells with siRNA against PD-L1 resulted in an increased decrease of cell viability by roughly 10% compared to T cell incubation alone [21].

As a more complex formulation, a triblock polymer of PEI-poly-caprolactone-PEG (PEI-PCL-PEG) with FA as a targeting moiety coupled to PEG was developed (Fig. 1a) [43]. PCL yielded biodegradability and, due to its hydrophobic nature, was used as the hydrophobic core of the self-assembled nanoparticles (Fig. 2c). The PEI-PCL-PEG triblock polymer retained siRNA better than PEI in the presence of heparin, demonstrating an improved encapsulation efficiency. In contrast, the triblock polymer showed a more efficient heparin-induced release at pH 4.5. According to the authors, this indicates that siRNA release in endosomes was higher compared to PEI nanoparticles. An siRNA against toll-like receptor 4 (TLR4) was used in this study in combination with FA conjugated. Previous studies had suggested that upregulation of TLR4 results in chemoresistance, which was reversed by siRNA in this study [43–46].

Zou et al. used a similar polymer, albeit with a different arrangement of the polymer blocks (FA–PEG–PEI–PCL) combined with a chemotherapeutic next to siRNA [47]. Bel-2 was targeted in an effort to re-sensitize cells to doxorubicin. Bel-2 is an apoptotic inhibitor that is upregulated in ovarian cancer, which results in decreased sensitivity to most cytotoxic agents [48]. Doxorubicin was incorporated into the nanoparticles in order to reduce side effects. Furthermore, to decrease the cationic toxicity of PEI, low molecular weight linear PEI instead of high molecular weight hyper-branched PEI was used.

Li et al. used a PEG-PLL-PEI triblock polymer in which PCL was replaced by poly-γ-l-lysine (PLL) [22]. Like PEI, PLL has a high charge density that can be used for siRNA complexation. However, similar to PCL, PLL is biodegradable thereby increasing the degradation of the ternary copolymer. In this case the inhibitor of apoptosis protein (XIAP) was targeted, which is upregulated in ovarian cancer SK-OV-3 cells [22]. To enhance selective delivery, a trastuzumab single chain antibody that targets the HER2/neu receptor was added. In spite of the fact that this receptor is overexpressed in SK-OV-3 cells, the number of HER2/neu positive ovarian tumors is low and its prognostic significance is controversial [22,49–51]. The targeted nanoparticles showed enhanced tumor uptake in nude mice with a subcutaneous tumor [22]. Still, the majority of the nanoparticles distributed to the liver. Nevertheless, the tumor showed necrosis in mice that were treated with nanoparticles containing siRNA against XIAP at N/P 20, while the other organs were unaffected. 90% of the mice in the targeted group survived at 8 weeks, compared to 50% survival for controls. The benefit was also shown to be greater in combination with chemotherapy than chemotherapy alone [52].

### Table 5

<table>
<thead>
<tr>
<th>Article</th>
<th>NP composition</th>
<th>Size (nm)</th>
<th>Zeta potential (mV)</th>
<th>siRNA target</th>
<th>Chemotherapeutic</th>
<th>Cell line in vitro</th>
<th>Murine model</th>
<th>Cell line in vivo</th>
<th>Tumor site Drug administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shen [86]</td>
<td>DOPC NPs in silicon microparticles</td>
<td>1000 × 400 (30–35 liposome)</td>
<td>500–600</td>
<td>SK-OV3ip2, HeyA8-MDR</td>
<td>IP paclitaxel or docetaxel</td>
<td>Female nude BALB/c</td>
<td>SK-OV3</td>
<td>IP</td>
<td>SC for xenograft formation, intravagin for experiments</td>
</tr>
<tr>
<td>Chen [85]</td>
<td>M-MSN-PEG-KALA</td>
<td>160</td>
<td>23</td>
<td>VEGF</td>
<td>None</td>
<td>None</td>
<td>None</td>
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<td>None</td>
</tr>
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</table>

### Table 6

<table>
<thead>
<tr>
<th>Article</th>
<th>NP composition</th>
<th>Size (nm)</th>
<th>Zeta potential (mV)</th>
<th>polymer:siRNA ratio (w/w)</th>
<th>Targeted receptor</th>
<th>siRNA target</th>
<th>Chemotherapeutic</th>
<th>Cell line in vitro</th>
<th>Murine model</th>
<th>Cell line in vivo</th>
<th>Tumor site Drug administration</th>
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<tr>
<td>Florinas [91]</td>
<td>PCE-albumin-ABP</td>
<td>3001</td>
<td>−34.6 ± 5.4</td>
<td>10:1</td>
<td>None</td>
<td>VEGF</td>
<td>None</td>
<td>None</td>
<td>N/A</td>
<td>Female nude mouse</td>
<td>N/A</td>
</tr>
<tr>
<td>Florinas [92]</td>
<td>PCE-albumin-ABP</td>
<td>3001</td>
<td>−34.6 ± 5.4</td>
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<td>None</td>
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<td>None</td>
<td>N/A</td>
<td>Female nude mouse</td>
<td>N/A</td>
</tr>
</tbody>
</table>

PCE, perfluorocrownether; ABP, arginine grafted polymers; VEGF, vascular endothelial growth factor; N/A, not available – information not included in the corresponding article; SC, subcutaneous; IT, intratumoral.
group survived > 45 days, whereas none of the control animals survived this long.

Another triblock approach for combination therapy of siRNA and doxorubicin was based on reducible micelles consisting of PEG-pAsp (AED)-PDPA [52]. Doxorubicin was loaded into the pH sensitive poly(2-(disopropyl amino)ethyl methacrylate) (PDPA) core. siRNA against Bcl-2 was loaded in the reduction sensitive middle layer of poly(N-(2,2'-dithiobis(ethylamine)) aspartamide (PAsp(AED)), and PEG formed the corona that stabilized the particles. Because these nanoparticles did not contain PEI, they relied on a different mechanism to induce endosomal escape. The particles were designed to be stable in the bloodstream but to disassemble and release their contents at low pH and high glutathione concentrations as present in lysosomes and cytosol, respectively [52]. In vitro experiments showed that the particles indeed released their contents at a pH of 5.0 and high glutathione concentration (Fig. 2a). Biodistribution experiments revealed that particle uptake occurred mainly in the tumor and liver and that they were retained there for at least 24 h. Clear synergistic effects of siRNA and doxorubicin were shown in vitro and in vivo. However, only the tumor and no other organs was assessed for structural abnormalities and apoptotic markers after the in vivo experiments.

2.2. Polysaccharide based nanoparticles

Chitosan is a natural cationic polysaccharide that is composed of D-glucosamines and can readily form nanoparticles through electrostatic interactions with oligonucleotides (Table 2, Fig. 1a) [53]. Li et al. used these features to create nanoparticles that consisted of chitosan oligosaccharide lactate (COL) in combination with tripolyphosphates (TPP) as a counterion [23]. The particles remained highly cationic despite the counterion, which resulted in interaction with red blood cells, opsonization, and activation of the immune system that led to

![Fig. 1. Examples of the drug delivery systems that are discussed in this review. A Polymeric nanoparticles, with a triblock copolymer on the left and a chitosan based nanoparticle on the right. A triblock copolymer typically consists of a hydrophobic unit followed by a corona of a hydrophilic unit. This part is mostly cationic and can bind siRNA through electrostatic interactions. The outermost layer consists of a polymer, such as PEG, that shields the nanoparticles from the immune system and prolongs circulation time. Chitosan nanoparticles are less organized and form an amorphous complex of polymer chains to which siRNA binds through electrostatic interactions. B Lipid based nanoparticles with a liposome on the left and a lipid nanoparticle (LNP) on the right. Liposomes consist of a lipid bilayer and siRNA is entrapped in the aqueous core. Variation of the phospholipid composition alters the net charge and stability of the liposome. Also, compounds like cholesterol are often added to increase stability. LNPs consist of (inverted) micelles that co-aggregate with siRNA. C Dendrimeric nanoparticles are composed of a core from which branched polymers extent outwards from several branching points. Each additional layer is called a generation. D Mesoporous silica nanoparticles are large particles with pores in which different, smaller nanoparticles can be loaded. In this example, lipid particles are loaded into the pores. E Microbubbles are gas filled nanoparticles with a shell that can consist of a range of different materials. When ultrasound is applied, the bubble bursts, and the polyplexes that encapsulate siRNA will enter the cells through temporal perforations in the plasma membrane and microjets that forces the siRNA into the cells.](image-url)
elimination of the nanoparticles. To reduce surface charge and thereby increase circulation time, the nanoparticles were PEGylated. Targeting to ovarian cancer cells was achieved by functionalizing PEG with FA yielding FA-PEG-COL that was used to form nanoparticles containing siRNA against the hypoxia-inducible factor-1α (HIF-1α). Expression of HIF-1α is often higher in ovarian cancer and is associated with various aspects of cancer progression, like angiogenesis, cell migration, proliferation, survival, metastasis, and drug resistance and could therefore be a prognostic marker [54].

Gharpure et al. also used TPP chitosan with an siRNA against the Enhancer of Zeste Homolog 2 (EZH2) [55]. Overexpression of EZH2 is associated with a more proliferative and aggressive phenotype [56]. In this study, TPP chitosan siRNA nanoparticles were combined with docetaxel-loaded PLGA-PRINT (PLGA -Particle Replication In Non-wetting Templates) nanoparticles. PRINT is a method based on soft lithography that enables adjustment of particle parameters like size, shape and composition [57]. This formulation allowed for metronomic dosing of docetaxel. In metronomic dosing, the chemotherapeutic is administered more frequently in a lower dose to obtain a more constant concentration [58]. The PLGA-PRINT nanoparticles were rod shaped with a size of 80 × 320 nm and a polydispersity index of 0.159. The authors tested metronomic dosing by IP injections of either free docetaxel or docetaxel loaded nanoparticles in tumor bearing mice. In a dose finding study where siRNA was not included, the experimental group received 0.5–2 mg/kg docetaxel loaded PLGA-PRINT nanoparticles 3 times per week and the control group received 20 mg/kg free docetaxel every two weeks. The tumors of the mice treated with PLGA-PRINT were slightly but significantly smaller than those of the control group [55]. Subsequently, a combination of docetaxel loaded PLGA-PRINT nanoparticles with siRNA containing TPP chitosan nanoparticles was studied in vivo. A decrease in tumor weight was shown for the combination therapy, compared to loaded PLGA-PRINT and TPP chitosan nanoparticles alone. Unfortunately, controls with free docetaxel and free docetaxel with TPP chitosan siRNA nanoparticles were not included in these experiments.

Polylactic acid (PLA) can also be combined with chitosan. Babu et al. used PLA as an inner core loaded with cisplatin [59]. The outer layer consisted of chitosan ionically complexed to plasmid DNA coding for the proteasome subunit beta type-5 (P SMB5) and siRNA against sequestosome-1 (SQSTM1) (Fig. 2c). This combined delivery was used as proof-of-concept for the hypothesis that simultaneous upregulation of P SMB5, and downregulation of SQSTM1 would restore drug sensitivity in cisplatin resistant cells. The authors concluded that additional research on other cell lines had to be performed prior to in vivo studies with xenograft models [59].

While most polysaccharide nanoparticles were based on chitosan, Kobayashi et al. utilized dextran functionalized with thiol, PEG, and a hexylamine or octylamine tail [60]. Hexylamine-dextran was selected for encapsulation of doxorubicin and octylamine-dextran was used for production of nanoparticles encapsulating siRNA. Doxorubicin encapsulation was supposed to occur by hydrophobic interactions of the aromatic groups of doxorubicin with the triazol ring present on the click chemistry-modified dextran-hexylamine polymers. Higher toxicity of doxorubicin nanoparticles was shown with an MTT assay and knockdown of P-gp was confirmed by western blot, but unfortunately, the combination of both treatments was not studied [60]. Hence the synergistic effect of a combination therapy is unknown.

### 3. Lipid based nanoparticles

Most lipid based nanoparticles used for siRNA delivery are lipid nanoparticles (LNPs) and liposomes (Fig. 1b). Liposomes either shield healthy cells from a toxic compound, as illustrated by PEGylated liposomal doxorubicin (Doxil, Caelyx), or protect encapsulated cargo from degradation and enhance cellular uptake as for oligonucleotide delivery (Table 3). Liposomes can vary greatly in their phospholipid composition and additional
functionalities for targeting, shielding, etc. can be attached via lipid anchors [61–63]. Preferentially, cationic liposomes are used for intracellular delivery of oligonucleotides since these particles readily interact with the negatively charged plasma membrane of the cell.

In the context of ovarian cancer, Zhao et al., studied a cationic liposome consisting of a cholesterol derivative N-(cholesterylhexamisssuccinyl-amino-3-propyl)-N, N-dimethylamine (DMAPA) containing 52.5 mol% of the phospholipid DOPA, and 47.5 mol% of cholesterol [64]. This formulation was shown to be more toxic than other cationic liposomes [65,66]. The transmembrane protein NOTCH1, which is a known oncogene [67], was targeted with siRNA in this study [64]. The DMAPA-cholesterol containing nanoparticles were almost nontoxic in N/P ratios up to 120, and downregulation of NOTCH1 was roughly equal in DMAPA-cholesterol transfected cells compared to Lipofectamine control. Furthermore, a small decrease in cell viability after incubation with NOTCH1 siRNA compared to incubation with control siRNA was observed.

A study by He et al., used a more complex drug delivery system that was based on a zinc bisphosphonate nanoscale coordination polymer (NCP) containing 48 wt% cisplatin instead of an aqueous core [68]. This core was surrounded by a lipid bilayer of 1,2-dioleoyl-sn-glycero-3-phosphate (DOPA), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), and 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-PEG2000 (DSPE-PEG2000) [69]. These particles did not provide an aqueous core for the incorporation of siRNA, the cationic phospholipid DOTAP was used to improve cellular uptake and to retain the siRNA in the corona of the nanoparticle. siRNAs against Bcl-2, P-gp and survivin were combined to synergistically enhance the effect of cisplatin. Cisplatin was released through high intracellular cysteine and glutathione concentrations. Multiple cell lines were employed to analyze the in vitro effects of the nanoparticles, and SW-48-3 cells were used to establish a subcutaneous xenograft. Although biodistribution was not studied systematically, kidney and liver did not show any structural damage after treatment with the nanoparticles. The tumor, however, showed increased apoptosis and was much smaller in the treatment group compared to the control groups [68]. In vivo, only the synergistic effect of the combined siRNAs was assessed without single siRNA controls.

Besides liposomes, other lipid based delivery systems have been studied. siRNA was modified with an N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) group at the 3’ end of the sense strand and reversibly conjugated via a disulfide linkage to 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (PE-SH) incorporated into PEG2000-phosphatidylethanolamine (PEG2000-PE) based micelles [69]. These nanoparticles were subsequently used to assess effectiveness of a combination therapy of an siRNA against survivin with paclitaxel in a single nanoparticle [69,70]. Survivin is a member of the IAP family that is involved in MDR and apoptosis inhibition and is upregulated in most human tumors [68,71]. Disulfide conjugation stabilized siRNA against degradation in vitro [69]. In mice, both siRNA alone and siRNA / paclitaxel combination therapy showed the most significant decrease in survivin protein expression as analyzed with immunohistochemistry. Combination therapy furthermore resulted in the lowest tumor weight and volume [70]. Interestingly, nanoparticle treatment with siRNA against survivin without the addition of paclitaxel also led to a significant decrease of tumor volume. The results of this study were mainly obtained from animal studies without extensive in vivo data to mechanistically support the evidence derived from the xenograft models.

Shazad et al. developed reconstituted high-density lipoprotein (rHDL) solid lipid nanoparticles [72]. With a diameter of 12–18 nm, these particles were in particular remarkable for their small size. HDL is an important component of the lipid transport system and promotes transport of excess cholesterol to the liver for elimination [73]. Reconstituted HDL (rHDL) particles did not contain all elements of natural HDL, but consisted of apolipoprotein A-1, phosphatidylycholine, cholesterol, and cholesteryl esters. HDL particles are known to escape the reticuloendothelial system, resulting in a longer circulation time than standard cationic lipid formulations [74]. In addition, HDL particles are internalized into cells via scavenger receptor class B type 1 (SR-B1).

This receptor is mainly expressed in the liver and on most malignant cells and was therefore selected as a starting point for targeted drug delivery [74]. In mice, the nanoparticles were mainly taken up by the tumor and the liver in agreement with the pattern of receptor expression. Therapeutic efficacy was demonstrated by targeting focal adhesion kinase (FAK) or signal transducer and activator of transcription 3 (STAT3), which are involved in tumor development and progression [74]. Unfortunately, target gene expression was not assessed in the liver tissue. But liver function tests (ASAT/ALAT) were performed and these did not show liver damage [72].

Matsui et al. used Multifunctional Envelope-type Nano Device (MEND) lipid nanoparticles that contained the pH-sensitive cationic lipid YSK05. This formulation was used to deliver anti-Cd45 siRNA to peritoneal macrophages (PEMs) [75]. In ovarian cancer, PEMs create a pro-tumor niche by modifying the tumor microenvironment. As a consequence, these cells are a potentially interesting therapeutic target [76]. Particle uptake in PEMs increased 5.5- and 9.2-fold when particle size increased from 75 nm to respectively 200 and 340 nm. Such an increase was not observed in other cell types. Enlarging the MEND nanoparticles to 460 nm, however, did not further increase uptake and even decreased it slightly. IP administration resulted in the most effective downregulation of Cd45 since larger nanoparticles were unable to reach PEMs when they were injected IV [75].

Goldberg et al. developed large nanoparticles consisting out of lipiodoids and siRNA against the DNA repair enzyme poly(ADP-ribose) polymerase 1 (PARP1). Lipiodoids are lipid-like materials consisting of an amine-containing backbone out of which aliphatic chains extent that form structures similar to lipids and dendrimers [77,78]. PARP1 knockdown is effective in BRCA1 deficient cells since it induces extreme genomic instability, causing cell cycle arrest and cell death [77]. The authors hypothesized that amines in the lipiodoids bind siRNA via electrostatic interactions and that these structures facilitate endosomal escape upon acidification of the lysosomal compartment. Furthermore, they proposed that the hydrophobic chains interact with membranes, thus aiding endosomal release [77]. Unfortunately, these hypotheses were not addressed in this manuscript. The exact mechanism behind the higher effectiveness of these nanoparticles is therefore unknown. Despite that shortcoming, the authors clearly showed that mice bearing a BRCA1 deficient tumor that was treated with siRNA against PARP1 have an extended life span compared to control mice and mice bearing a BRCA wild type tumor [77].

4. Dendrimer based nanoparticles

Dendrimers are tree-like polymers of repetitively branched molecules that consist of a core from which branched layers (generations) of polymer extend outwards (Fig. 1c) [79]. Most dendrimers are based on amine structures like polyamidoamine (PAMAM), and multilayered dendrimers consisting of several functional units are common nanoparticle forming macromolecules (Table 4).

A generation 6 PAMAM dendrimer with a triethanolamine (TEA)-core was used to form nanocomplexes via electrostatic interactions with siRNA targeting the serine/threonine kinase Akt, a well-known oncogene associated with tumor cell survival, proliferation, and invasion [80]. Co-administration of the 85 nm particles and paclitaxel reduced tumor size and mortality in tumor bearing mice. However, treatment was based on intratumoral injection and therefore not representative for a clinical setting of EOC where the challenge lies in targeting of micrometastases. Injecting all tumor nodules is not feasible in a clinical setting.

Engelberth et al. modified a dendrimer based on enzymatically synthesized glycogen (ESG) [81]. Quaternary ammonium groups were introduced via an epoxide ring opening reaction with glycidyltrimethylammonium chloride (GTMA). These polymers bound siRNA to superoxide dismutase 2 (Sod2) via electrostatic interactions. Based on unpublished observations that Sod2 is highly expressed in ovarian clear cell carcinomas,
the authors hypothesized that downregulation of this mitochondrial antioxidant enzyme may lead to increased susceptibility to chemotherapeutically induced redox damage. Protein downregulation with the ESG nanoparticles was demonstrated on western blot. RNAiMAX Lipofectamine mediated delivery was more effective in inducing protein downregulation. However, RNAiMAX also was more cytotoxic, although this experiment was only performed once. Unfortunately, the authors did not test the hypothesis if the downregulation of Sod2 indeed causes higher susceptibility to ROS production by chemotherapeutic agents.

Shah et al. used modified polypropyleneimine (PPI) dendrimers in which siRNA was complexed by electrostatic interactions and a cage of dithiol containing cross-linkers was used to stabilize the complex [82]. Aggregation was prevented by PEGylation and a synthetic gonadotropin-releasing hormone (GnRH) analogue was coupled for specific targeting to cancer cells. Paclitaxel was coupled to a similar PPI dendrimer via a biodegradable succinic acid spacer and co-administered with the siRNA nanoparticle. siRNA was released in the reductive environment inside the cell and targeted at downregulating CD44 [82]. As one of few studies, the authors analyzed both uptake and effectiveness in different organs. They showed that the majority of targeted nanoparticles ended up in the tumor, although this still represented < 50% of the nanoparticles. Furthermore, a significant amount reached the liver, kidneys, lungs, and heart [82]. With a targeting ligand, however, > 75% of the nanoparticles distributed to the tumor and only a minor fraction was retained in the liver. Targeted nanoparticles loaded with paclitaxel or with paclitaxel and siRNA showed greatly increased apoptosis in the tumor and decreased toxicity in other organs compared to untargeted nanoparticles and paclitaxel alone. Apoptosis was in this case analyzed by the enrichment of histone-associated DNA fragments per gram tissue. Combination therapy of paclitaxel and siRNA was the most effective in the apoptosis assay, and when the tumor volume was measured.

A radically different approach was developed by Huang et al., in which DNA-based dendrimers were coupled to folate for targeting, and hybridized to siRNA against the mRNA-binding protein HuR. HuR stabilization mRNA molecules and influences translation of growth-related mRNAs that are important for multiple stress induced survival pathways [83,84]. Multiple signaling pathways were affected by the siRNA by this broad approach. Biodistribution was analyzed after IV injection of the nanoparticles, whereas efficacy was studied after IP injection. Efficient delivery and increased median survival from < 30 days to roughly 45 days was demonstrated in a xenograft mouse model. In spite of this success, the therapy was not sufficient to eradicate the whole tumor and cure the animals. Therefore, the authors proposed to combine an siRNA against HuR with chemotherapy or another siRNA [83].

5. Silicon based nanoparticles

The aforementioned formulations can be loaded into mesoporous silicon nanoparticles (MSNs) in order to achieve slow and sustained release of siRNA (Fig. 1d). These silicon particles are several micrometers in size, and have pores ranging from 1 to 50 μm (Table 5) [85,86].

In a study of Shen et al., MSNs were loaded with dioleoyl phosphatidylethanolamine (DOPE) based liposomes containing siRNA against the ephrin receptor A2 (EphA2) [86]. This epithelial protein-tyrosine kinase receptor is often overexpressed in ovarian cancer and is associated with increased angiogenesis, metastasis, and decreased survival [87,88]. “Nanoliposomes” were prepared by reconstituting a lyophilized mixture of siRNA and lipids with water. The resulting particles were retained in the 40–65 nm pores of discoidal porous silicon particles of roughly 1000 × 400 nm in size, creating a multistage vector system. The silicon particles were modified with 3-aminopropyltriethoxysilane (APTES), which converted the surface charge from negative to slightly positive. This modification also enhanced loading of the neutral to slightly anionic liposomes. Despite of their size, tumor accumulation of these MSNs was still observed which was attributed to the discoid shape [86,89]. The authors claim that multiple MSNs were detected inside the cells even though only the siRNA was fluorescently labeled. siRNA induced EphA2 knockdown for up to 9 days. Western blot, however, showed that a scrambled siRNA also knocked down EphA2 expression at this time point [86]. In the biodistribution study, the MSNs and the siRNA were separately labeled. Over a course of 12 h to one week, fluorescence from MSNs could be detected in the kidney and the liver, whereas fluorescence arising from the tumor disappeared within 24 h. The authors suggest that high fluorescence in the kidney resulted from the cleavage of the fluorescent dye from the MSNs [86]. The labeled siRNA on the other hand, was mainly found in the tumor and the kidney, and could still be detected above background after one week. The authors propose that the MSNs were stored in the liver as a depot and that the liposomes were released from there from where they reached the tumor by the EPR effect [86].

MSNs can also be loaded with naked oligonucleotides. Chen et al. used magnetic mesoporous silica nanoparticles (M-MSN) consisting of an Fe3O4 core and a mesoporous silica shell that showed a large loading capacity of siRNA against VEGF [85]. Since both siRNA and M-MSN particles were negatively charged, siRNA adsorption was only efficient in a strongly dehydrated environment [90]. After loading, the particles were coated with PEI to enhance oligonucleotide binding in an aqueous solution. This prevented siRNA from premature desorption [85,90]. Furthermore, PEG-KALA, where KALA refers to the peptide WEAKLA-KALAKALAKHLAKALAKLACEA, was conjugated to the particles, which improved cellular uptake and promoted escape of the nanoparticles from lysosomes [85]. The superparamagnetic Fe3O4 core of the nanoparticle was also used as a contrast agent for magnetic resonance imaging (MRI). Due to this theranostic combination of functionalities, these MSNs provide the possibility to determine and optimize the dose and effectiveness in clinical applications [85].

6. Microbubble based nanoparticles

Microbubbles in combination with ultrasound are a strategy for stimulus-triggered drug release. Microbubbles consist of a gas filled core with a shell that can be composed of proteins, surfactants, lipids, polymers, or a combination thereof (Fig. 1e) [91]. Similarly to MSNs, microbubbles are typically much larger than nanoparticles and can be as large as red blood cells [91]. Microbubbles are often used as contrast agents for imaging, but they can also be employed as drug delivery vehicles (Table 6). Because oligonucleotides do not dissolve in gas, they are incorporated in the shell. Upon ultrasound exposure at the target site, the gas core starts to oscillate, which results in disruption of the shell. This mechanism induces uptake in two, mutually enhancing ways. First, the ultrasound temporarily perforates cells, which allows the entrance of siRNA. Second, the force with which the bubble bursts causes microjets that force the siRNA directly into the cells [92,93].

Microbubbles of 3 μm in diameter with an albumin shell and a perfluorocrownether (PCE) gas core were loaded with an arginine grafted polymer (ABP), a disulfide linked polyamidoamine backbone with arginine functionalities. These ABPs formed stable polyplexes of < 200 nm with siRNA directed against VEGF, and loading occurred through electrostatic interactions of the anionic microbubbles and cationic ABPs [93]. The arginine residues increased transfection efficiency and endocytosis of the polyplexes. siRNA was released into the cytosol when the disulfide amine backbone was reduced by intracellular glutathione [93,94]. These nanoparticles showed higher transfection efficiency than 25 kDa branched PEI. In 2014, Florinas et al. confirmed improved siRNA uptake in vivo in a subcutaneous murine ovarian cancer model [92]. A subcutaneous xenograft was used in this study and an ultrasound probe was placed directly on the tumor. Unfortunately, this method is not clinically feasible since ovarian cancer resides in the abdominal cavity. The ultrasound should in this case either be applied to the whole abdomen or to every individual
metastasis, and this should also include small tumor deposits invisible to the naked eye.

Ultrasound triggered lipid microbubbles have also been applied for GnRH and FRα targeted intraperitoneal delivery of paclitaxel [95,96]. Effectiveness was assessed in an IP xenograft model. However, the whole abdomen was subjected to ultrasound. This could also induce paclitaxel release from the microbubbles that are not bound to the tumor, thereby reducing specificity. It therefore remains to be shown to which degree this approach is also viable for the systematic eradication of cancer micrometastases in the much larger human abdominal cavity.

7. Discussion

7.1. Nanoparticle design

A large diversity of carriers and cargos has been explored for siRNA delivery to ovarian cancer. Nearly all approaches have in common that they incorporate the anionic siRNA via non-covalent complexation with the cationic carrier. Furthermore, the net positive charge of these particles also promotes cellular uptake. The complexation method was different in only three studies. In the first study, PEG2000-PE micelles were reversibly conjugated to a modified siRNA against survivin [70,97]. The second study utilized DNA based dendrimers that hybridized siRNA to one of the single-stranded sequences of the dendrimer [83], and the third study used mesoporous silica nanoparticles in which naked siRNA was loaded under hydrophobic conditions [85].

Starting from the fundamental design of a non-covalent poly-/lipoplex, diversity and complexity are introduced through (i) structural variation of the carrier and inclusion of shielding in the form of PEGylation, (ii) incorporation of targeting modalities, (iii) variation in size, and (iv) means to promote intracellular disassembly. Two more variables are introduced through variation of the siRNA target and the combination of siRNA with standard chemotherapeutics. These latter two variables are not fully independent of one another as some siRNAs target genes to achieve a resensitization of cancer cells to chemotherapy.

Many formulations rely on a high N/P ratio to incorporate siRNA into the particles. An excess of amines results in a net positive charge of the particle. Fundamental for the evaluation of a carrier is the balance between activity and toxicity in many cases, increased uptake efficiency comes along with a higher risk of toxicity as is shown for PEI based siRNA delivery. Shielding in the form of PEGylation is therefore a common method to reduce this cytotoxicity. Unfortunately, the effects of siRNA delivery and nanoparticle toxicity in healthy tissues are often not studied well. Some articles investigate structural damage on histological sections, but this is only a crude assessment of the damage. The effects of cationic damage and protein downregulation by siRNA can be more subtle and this should therefore be assessed in healthy organs as well as in the tumor [98].

In order to enhance specific tumor cell targeting, targeting modalities are often incorporated into the nanoparticle formulation. A common receptor for active targeting in ovarian cancer is the folate receptor alpha. FRα is overexpressed on the majority of ovarian tumors, and can be targeted with antibodies or FA [23,38]. However, following IP injection entry of intact delivery vehicles into systemic circulation has to remain limited since the receptor is also present in healthy tissues. Several FRα targeting candidate drugs are being tested in clinical trials [37], but unfortunately, some have failed despite initial promising results [99].

Independent of the presence of targeting ligands, accumulation of nanoparticle formulations for EOC therapy in the liver is a concern even after IP administration [26,52,68,82,86]. Following IP administration, Shah et al. saw < 50% of the polypropyleneimine (PPI) dendrimers accumulating in the tumor and > 25% in the liver [82]. Adding an active targeting ligand against GnRH improved biodistribution and resulted in a tumor accumulation of > 75%. Also, apoptosis was much higher in the tumor than in the liver and kidneys. Shahzad et al. also observed significant liver uptake of rHDL particles targeting scavenger receptor B1. However, they did not observe significant differences in biodistribution and uptake when IV and IP administration were compared [72]. This included the tumor and the liver. It should nonetheless be noted that the hepatic accumulation seemed to be higher upon intravenous administration compared to intraperitoneal administration. Even though this was not a significant increase according to the microscopy image analysis.

Polymer and lipid based formulations vary in size between 20 and 250 nm. Formulations such as microbubbles and MSNs on the other hand, are much larger and can reach sizes of up to several micrometers. A larger size can be a preferred characteristic for IP therapy of ovarian cancer since larger particles are expected to be retained in the intraperitoneal cavity for quite some time before entering the blood stream. Matsui et al. compared IV with IP administration in dependence of particle size [75]. They concluded that particles with a size of 75–340 nm were able to transfact macrophages in the peritoneal cavity after IV injections. However, nanoparticle uptake in these cells corresponded to only 0.1% of the total injected dose and most of the particles ended up in the liver. The authors furthermore showed that IP injection achieved efficient gene knockdown at an siRNA dose that was 330 times lower compared to IV administration [75]. However, in ovarian cancer, the intraperitoneal cavity can be filled with ascites, a protein and lipid rich fluid that contains blood and tumor cells that may sequester particles and thus limit bioavailability [100]. Also, nanoparticle availability could be restricted by the extracellular matrix and absence of entry into the tumor-perfusing vascularization [101]. Therefore, systematic studies are required to better understand to which degree nanoparticles can enter the tumor stroma from the peritoneal side.

As intraperitoneal retention increases with size, the use of micro-meter sized silicon particles can be interesting since these combine long half-life with their slow-release ability. Under physiological conditions, MSNs are degraded into silicic acid, which is eliminated by the kidney or deposited into connective tissues [102]. Silicon is the third most abundant trace element and ortho-silicic acid is linked to some therapeutic effects [103]. This indicates that low levels of silicon will probably not cause cytotoxic effects.

7.2. siRNA design

Two types of siRNA targeted pathways can be discriminated: Those relating to cell resistance and cell survival, and those relating to tumor growth and angiogenesis (Fig. 3). The most common targets in EOC therapy are MDR genes and genes involved in cell survival, MDR1, Bcl-2 and survivin (Tables 1–6). Downregulation of these genes resensitizes the cells to chemotherapy and siRNAs targeting these pathways are always combined with chemotherapeutic treatment. This combination has been applied in almost half of the discussed studies and in all nanoparticle formulations except microbubbles (Tables 1–5). One exception in which siRNA against MDR1 was tested without the addition of chemotherapy is the publication of Kobayashi et al. [60].

VEGF and FAK are the most common targets regarding cell migration, proliferation, and angiogenesis. Other prominent siRNA targets were CD44 and EphA2 that are both involved in angiogenesis. Interestingly, CD44 was more often used as a receptor for active targeting than as an siRNA target. Two studies utilized a combination of more than one siRNA. Both contained an siRNA directed against MDR1. He et al. also targeted Survivin and Bcl-2, whereas Talekar et al. addressed PKM-2 [26,68]. These strategies aimed at reducing the chance of survival through upregulation of escape pathways. Furthermore, one study combined siRNA therapy against the ubiquitin binding protein P62 with transfection of a plasmid that encoded for the β5 subunit of the 26S proteasome complex, causing an interplay between restored β5 function and P62 knockdown that resensitized cells for cisplatin [59]. In addition, some studies aimed at reducing the systemic toxicity of
chemotherapeutics by also encapsulating them in nanoparticles with or without a targeting moiety (Tables 1–6).

At this point it is still not clear which (combination of) targets are the most promising for clinical application. Clearly, some targets, for example HER2/neu, seem less likely to succeed due to low expression in ovarian cancer [49].

### 7.3. Clinical applicability

For clinical application, reproducibility and cost efficiency are also considerations when designing new formulations. These boundary conditions favor simple designs that incorporate the minimal set of functionalities for achieving a therapeutic benefit. Interestingly, there was nearly no cross-referencing between different approaches in the reviewed literature. Therefore, also the benefit of an additional functionality is difficult to assess.

Liposomes are expensive to manufacture and oligonucleotides need to be encapsulated in the form of polymer-complexed nanoparticles. The potential advantage is the versatile surface functionalization. However, LNPs provide very similar possibilities with a more straightforward overall design (Table 4). Polymer-based nanoparticles combine a good shelf life with sustained release but this may also restrict the bioactive concentration. No polymer-based oligonucleotide formulation has made it into the clinic so far, even though PEI toxicity can be decreased by the addition of PEG and less toxic alternatives such as PAMAM-based polymers have been explored. As mentioned above, mesoporous silica nanoparticles are an interesting option for taking advantage of retention in the intraperitoneal cavity and for inducing sustained release of bioactive nanoparticle formulations. Moreover, ultrasound-triggered microbubbles overcome problems such as siRNA entrapment and inefficient release from the delivery vehicle, which are common to polymer-based delivery systems and liposomes. However, the effectiveness of this approach for the large human peritoneal cavity remains to be shown.

### 7.4. Methodological considerations

Methodological differences of the presented studies also complicate the identification of the most promising system for clinical use. The large majority of studies based their conclusions on tests with a single

**Fig. 3.** Receptors for active targeting and siRNA targets discussed in this review. Receptors for active targeting are shown on top. Several targeting moieties were used to induce cell specific nanoparticle uptake. Some active targeting strategies incorporated the respective ligand such as folate for the folate receptor and GnRH for the GnRH receptor. Antibodies have also been used widely, and sometimes the inherent properties of the nanoparticle formulation showed preference for certain receptors. Hyaluronic acid for example binds to CD44, and rHDL nanoparticles are taken up by the Scavenger-Receptor B1. All siRNA targets are shown inside the cell and on the bottom part of the plasma membrane. All intracellular targets are shown as rounded rectangles. Ellipses relate to factors that link these targets. These were included to indicate the connection of various pathways. Several studies interfered with apoptosis inhibiting pathways (yellow). Others addressed the PI3K/AKT/mTOR pathway (green), DNA repair and replication (blue), and cellular metabolism (pink). Some studies intended to alter the tumor microenvironment by downregulating certain receptors like VEGF, CD44, TLR4, and EphA2. Also, several studies down regulated P-gp expression in order to overcome drug resistance, and PD-L1 was targeted in order to induce an immune reaction. Akt, protein kinase B; CLDN3, claudin-3; EphA2, ephrin receptor A2; FAK, focal adhesion kinase; GnRH, gonadotropin-releasing hormone; HER2/Neu, receptor tyrosine-kinase erbB-2; HIF1a, Hypoxia-inducible factor 1-alpha; HuR, ELAV-like protein 1; mTOR, mechanistic target of rapamycin; P62 / SQSTM1, polyubiquitin-binding protein P62 / sequestosome 1; PARP1, poly ADP ribose polymerase 1; PDK1, Pyruvate dehydrogenase lipoamide kinase isozyme 1; PD-L1, Programmed death-ligand 1; P-gp, p-glycoprotein; PI3K, Phosphatidylinositol-4,5-bisphosphate 3-kinase; PKM2, Pyruvate kinase isozymes M2; SOD2, Superoxide dismutase 2; SR-B1, scavenger receptor B1; STAT3, Signal transducer and activator of transcription 3; TLR4, toll like receptor 4; VEGF, vascular endothelial growth factor; XIAP, X-linked inhibitor of apoptosis protein.
cell line, whereas only a few studies used multiple cell lines to validate their drug delivery system. The most widely used cell lines are well characterized, so specific mutations are known. However, in spite of their frequent use, the cell lines SK-OV-3 and A2780 are poorly suited as models for high-grade serous ovarian cancer since their genetic fingerprint is very different from primary tumors [104]. Therefore, results obtained with these cells should be validated with more reliable ovarian cancer models. The likelihood that a therapy will be applicable in the clinic will generally increase when it is validated with various ovarian cancer cell lines.

Monolayer cell culture models can give fundamental information on the working mechanism of nanoparticles. These simple models, however, do not recapitulate the complex situation of a clinical tumor. They lack a 3D arrangement of cells, stroma, other cell types, and fluid flow. These factors can greatly alter the bioavailability and effectiveness of nanoparticles. Penetration of particles into tumor tissue can for example be compromised by size and capture in the extracellular matrix [105]. 3D tumor models could therefore help in making a better prediction on the effectiveness of a certain therapy as an intermediate step between simple in vitro and in vivo experiments.

Alternatively, primary solid gynecologic tumors and malignant ascites from ovarian cancer patients can be used instead of cell lines [82]. These model systems can provide a more realistic representation of the clinical setting. However, the use of primary tumor material results in heterogeneity between samples and even within one sample. Larger numbers of samples are therefore needed to obtain statistically reliable results.

In vivo, drug delivery systems were validated in murine models. Most studies used immunodeficient athymic nude BALB/c mice and two studies used severe combined immunodeficient (SCID) mice in order to establish a xenograft model [27,70]. Goldberg et al. used athymic nude BALB/c mice, but also immunocompetent FVB/NJ mice in which tumor growth was possible by utilizing a murine ovarian cancer cell line [77]. The authors did not report any differences in outcome between the immunodeficient and immunocompetent mice. A similar strategy was used by Huang et al. as they established a tumor model by injecting C57BL/6J (black six) mice with murine ovarian surface epithelium derived ID8-Fluc cells [83]. The last group to use immunocompetent mice studied macrophages in ICR mice without establishing a tumor altogether [75]. Immunodeficient mice have to be used to develop xenografts from human origin. Studying the interactions of novel drugs and the immune system is unfortunately not possible in these models. Knowledge of these interactions is nevertheless very important since other therapies such as chemotherapeutics are notorious for bone marrow depression. Furthermore, the majority of these tumors consisted of the questionable SK-OV-3 cells. Unfortunately, it has not been validated as to which degree immunodeficient animals reproduce the essential characteristics of EOC.

A large source of variation between in vivo models is the tumor location, which is either subcutaneous or intraperitoneal. A subcutaneous tumor has the advantage to be easily accessible and easy to monitor over time without sacrificing the animal. In contrast, an intraperitoneal model is more realistic though tumor morphology and tumor microenvironment may still be different from the human situation. For example, only OVCA-8 cells were able to form ascites after IP inoculation in a study that analyzed the in vivo growth of 11 different ovarian cancer cell lines [106]. Unfortunately, the only study utilizing OVCA-8 in this review used a subcutaneous tumor model [27]. Cells such as KURAMOCHI and OVSAHO cells are genetically closer to primary tumor tissue than SK-OV-3 cells [104]. However, these cells only grow in SCID mice and show a disseminated growth pattern as compared to SK-OV-3 cells which show oligometastatic growth [107]. Lastly, experimental bias can occur as, for example, animal randomization was not performed in most studies. If the guideline on the assessment of risk of bias in animal studies was followed, most experiments could be improved in order to make their results more valid [108]. A more systematic comparison of injection routes in murine models should also be instructive. An exception with very little predictive relevance is intratumoral injection of subcutaneous tumors as this neglects the highly disseminated nature of late stage ovarian cancer and is therefore not clinically relevant.

Astonishingly, in the field of siRNA delivery there has been little consideration as to which degree IP therapy requires characteristics that differ from those of other application routes. For example, to which extend does particle size affect extravasation into systemic circulation? To which degree is surface shielding required if there is no contact with the reticuloendothelial system? Are there differences in the requirements for degradation versus excretion? To which degree is the presence of targeted receptors such as the folate receptor on other cell types a concern, if entry into systemic circulation is highly delayed and/or reduced?

In conclusion, numerous delivery techniques, targets, and siRNAs have been studied in the context of EOC. All methods seem successful due to a positive publication bias and drug delivery systems have a tendency towards increasing complexity since many groups strive to continuously improve the characteristics of a specific delivery formulation in their own line of research. What is urgently needed is a critical cross validation of delivery approaches in a clinically relevant context. For example, the polymer-based formulations with the most beneficial activity to toxicity ratio need to be compared to LNPs for systemic and IP application. A further criterion should be the necessity for targeting and shielding. Activity in combination with simplicity should be the ultimate design rationale.

Declaration of interest

None.

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Appendix A. Supplementary data

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