FIGURE 3. Assessing the expression levels of protein LCP-1. The concentration of the LCP-1 was tested in a range of control and cancer cell lines before and after the treatment with EVmiR™ loaded with the miR-885-5p by using quantitative western blot assays.

FIGURE 4. Cell proliferation assay on breast cancer cell MDA-MB-231. Cell proliferation assay on MDA-MB-231 breast cancer in the presence of EVmiRs (LC: low concentration, MC: medium concentration and HC: high concentration), GSI inhibitor and DMSO as known cytotoxic factors as controls, and PBS/normal conditioned media as internal controls; cell treatment for 4 and 7 days.

FIGURE 5. *In vitro* experiments of MDA-MB-231 cells in treatment with EVmiRs confirmed their internalization and the induction of a biological effect as evidenced by membrane damage, cell shrinkage and blebbing in the targeted cells (at various time points such as Day 2, 5 and 7 of the treatment). Control cells with no treatment or with DMSO treatment are shown for comparison.

FIGURE 6. In vivo whole-body biodistribution assay. FIG.6A Labeled EVmiRs (DiD stained) and synthetic DiD-stained particles were administered to control mice. Labeled EVmiRs (DiD stained) were administered in tumor-bearing mice that were tumor-GFP-labeled. FIG. 6A shows co-localization of EVmiRs with the tumor site. Fluorescence has been surrounded by dashed lines. **FIG. 6B.** Animals were tested at different time-points for *in vivo* biodistribution using an in-house developed fluorescence imaging system. Co-localization of EVmiRs (fluorescence has been surrounded by dashed lines) with the tumor site (tumor has been surrounded by dashed lines in the left-handed panel) was evident for different time points.

FIGURE 7. Small image shows scanning electron micrograph of EVmiR and larger image shows the attack of EVmiRs against the MDA-MB-231 breast cancer cell line. EVmiRs co-localize with the tumor cells and load their contents though membrane uptake mechanisms.

SUMMARY

The present disclosure designs for delivery of a specific combination of miRNA sequences (including miR-885-5p) encapsulated in carriers of biological or synthetic origin (size 30-300 nm) containing chemotactic factors and proteins of stem cell origin (Table 2). The proposed construct titled EVmiR™ (Figure 1) will affect the action of a group of genes and proteins (including LCP1/LCP-1) associated with cancer initiation, growth, aggressiveness and metastasis in LCP-1 positive cancers (Table 1).

TABLE 2. The main components of the engineered EVmiR™ construct.

Carrier Components	
Proteins	P-selectin, Integrin beta-1, Vascular cell adhesion protein 1, Annexins, Tumor susceptibility gene 101 protein, Hsp70-binding protein 1, etc.
Cluster of Differentiation^{+}	CD9, CD29, CD63, CD73, CD81, CD90, etc.
Cluster of Differentiation^{-}	CD31, CD34, CD45, etc.
Endogenous miRNAs	miR-16-5p, miR-23a-5p, miR-125b-5p, miR-145-5p, miR-146a-3p, miR-181c-5p, miR-218-5p, miR-495-3p, let-7b-5p, etc.
Additional miRNAs (synthetic or *de novo*-induced)	miR-30a-5p, miR-30b-5p, miR-30c-5p, miR-30d-5p, miR-30e-5p, miR-194-5p, miR-302a-3p, miR-302a-5p, miR-335-3p, miR-335-5p, miR-367-3p, miR-373-3p, miR-885-5p, etc.

A therapeutic system based on specific miRNAs selected from a panel that includes the sequence miR-885-5p, and are loaded within biological, synthetic or chemical formulations including extracellular vesicles. This construct targets and modulates the expression of a specific group of oncoproteins (including LCP-1) that are associated with cancer initiation, growth, aggressiveness and metastatic potential in LCP-1 positive cancers. The successful delivery system is of a narrow size range (30-300 nm diameter) with stem cell associated properties of biological or synthetic origin. These vesicles act as targeted carriers of specific therapeutic miRNA sequences and once administered, migrate to tumor sites in order to fuse with, and deliver their therapeutic load directly into cancer cells.

In one aspect, the present disclosure refers to an extracellular vesicle (EV) loaded with a panel of miRNAs or with nucleic acid encoding the panel of miRNAs, wherein the panel of miRNAs comprises miR-16-5p, miR-23a-5p, miR-125b-5p, miR-145-5p, miR-146a-3p, miR-181c-5p, miR-218-5p, miR-495-3p, let-7b-5p, and miR-885-5p, wherein the EV is produced from a stem cell of mesenchymal origin modified to overexpress miR-885-5p as compared to a wild-type cell of the same type, wherein the EV selectively migrates to tumor tissue as compared to healthy tissue in a mammal.

In one aspect, the present disclosure refers to use of the EV disclosed herein, in the manufacture of a medicament for treating LCP-1 positive cancer in a subject.

PROBLEM: Cancer drugs currently in the market have yet to achieve the thorough eradication of tumors and the effective remedy of cancer disease in human patients. This is mainly due to difficulties in blocking the complex cancer growth and metastasis process (*therapeutic efficiency*) as well as due to inadequate delivery of therapeutic agents to tumor sites (*tumor targeting*). This lack of effective therapeutic options opens the door to new opportunities for novel approaches for targeted delivery of cancer therapy that can intervene in multiple cancer cell processes. Development of metastasis causes the most serious clinical consequences of cancer and is responsible for over 90% of cancer related deaths[14]. The process of cancer growth and metastasis is complex and our understanding of the molecular mechanisms that regulate them remain limited. The discovery of key targets in the oncology field will open new avenues for the fight against a broad range of cancer types such as melanoma, glioblastoma, ovarian, breast, cervical, colorectal and prostate cancer etc. Tumor cell migration and metastasis require

dynamic rearrangements of the actin cytoskeleton. Interestingly, LCP-1 is responsible for the organization of actin filaments and is also aberrantly expressed in the many types of blood cells. We have identified that the increased expression of this protein in epithelial cancers activates the metastasis pathway through the circulatory system allowing metastasis in distant sites in combination with other signature genes (Genes Panel, Table 1). Similarly, the identification of an ever-expanding list of oncoproteins that play a role, not only in metastasis, but also in initiation and growth of primary tumors linked to LCP-1 will open up a wealth of opportunities in the oncology field to design and develop novel cancer therapeutics that can target multiple stages of the disease.

SOLUTION: Tumor cell initiation, migration and metastasis require dynamic rearrangements of the cell cytoskeleton and the activation of key oncogenic pathways. Interestingly LCP-1 protein, originally described as a leukocyte specific protein, is aberrantly expressed in several malignant tumors and interacts with several other cancer-associated proteins such as the ones described in Table 1 (Protein Panel). Therefore, we have developed a panel of associated miRNAs (miRNA Panel, Table 1), and we have utilized it to design custom combinations of therapeutic miRNAs that can interfere with the expression of the above cancer associated proteins via targeting of their respective genes (see Genes Panel, Table 1).

In our disclosure, the therapeutic miRNAs are administered to tumors via a novel delivery system based on extracellular vesicles (EVs), although our panel of miRNAs can also be delivered with any other tumor-targeting carrier system (such as nanoparticle- or liposome- based systems). Thus, administration of our custom miRNA combinations described herein may be accomplished by any acceptable method that allows the miRNA or nucleic acid encoding the miRNA to reach its target. In a particular example of the disclosure, EVs have been specifically engineered to deliver our therapeutic miRNAs to tumor sites and inside cancer cells. The EVmiR™ complexes are engineered extracellular vesicles (EVs) of stem cell origin containing specific miRNA sequences that act as direct inhibitors of LCP-1. The EVmiR™ is also engineered to target and manipulate the expression of another group of oncoproteins that is directly involved in the LCP-1 pathway and mainly upregulated in solid tumors (Protein Panel, Table 1). The EVmiR™ complexes are ideal delivery vehicles as they retain the membrane receptors that allow stem cells to home selectively into tumor sites and target malignant cells, thus avoiding the targeting of healthy cells. The EVs home and engraft in solid tumors via specific chemokine receptors, fuse to tumor cell membranes, and incorporate miRNA directly into the target cancer cell, thus exerting their therapeutic effect while minimizing side effects associated with conventional therapies. These EVmiR™ complexes offer enhanced prophylactic and therapeutic potential due to their ability to target multiple molecules in malignant cells when compared with approaches targeting single genes and induce

immunosuppression through cytokine signaling inhibition making this approach especially suitable as component of therapeutic strategies in recurring cancer disease.

In our disclosure, we target, both *in vitro* and *in vivo,* the cancer related protein LCP-1 (as well as the oncoproteins in Table 1) via delivery of a combination of miRNAs encapsulated within the EVmiR™ or other appropriate vesicles.

We have constructed a novel cellular EVmiR™ complex containing a group of naturally occurring and synthetically overexpressed miRNAs (either by using synthetic premiRs, mature miRs, antimiRs or actively *de novo* induced miRs). The EVs are intact vesicles, with a size of 30-300 nm in diameter, formed from the plasma membrane and enriched in cytoplasmic components, cell surface proteins and bioactive phospholipids derived from the plasma membrane of their parent cells (see Table 2). This customized complex directly targets the expression of LCP-1 and other cancer related proteins (Table 1) that are specifically found in the tumor environment and which can block cancer processes such as tumor growth and metastatic progression. We have found that miRNAs associated with cellular particles such as our engineered EVs are protected from nuclease degradation and are able to be transferred in a variety of cells in order to prophylactically or therapeutically alter the gene expression of the recipient cells.

In certain examples, the panel may comprise naturally occurring miRNAs of stem cell origin and (one or more) synthetically overexpressed miRNAs, wherein the synthetically overexpressed miRNAs are synthetic premiRs, mature miRs, antimiRs or *de novo* induced miRs.

Our disclosure is applied prophylactically and therapeutically as part of a broader personalized medical strategy that includes appropriate patient selection as well as diagnostic, prognostic, therapeutic and monitoring components. The benefits of our disclosure, an improved pharmaceutical offering that has high selectivity and lower toxicity than earlier options, will be multifactorial. The cost of treating non-tolerated toxicities will be reduced and time of completion of active treatment will be significantly decreased due to the fact that toxic effects will not have to interrupt treatments. Optimized patient selection will be based on molecular profiling of the tumor and patient response variables paving the way for more efficient drug use and leading to lower mortality and morbidity rates. For this purpose, a companion diagnostic kit for assaying the levels of LCP-1 and the other oncoprotein targets, as well as monitoring the expression of our panel of miRNAs in tissue and/or liquid biopsies, has been developed. Thus, the EVmiR™ approach for targeted cancer therapy has been validated by utilizing the kit on clinical samples of tumors expressing the targeted oncoproteins and associated miRNA sequences.

ADVANTAGES: In the last 20 years, the oncology field has witnessed the introduction of a new class of drugs based on the concept of targeted therapy - drug treatments developed to selectively target genes,

proteins and signaling pathways that have been shown to play a role in cancer development. Several targeted therapies have shown efficacy in a wide range of cancers and more are being continuously introduced, driven by new knowledge in molecular biology and advances in chemical and molecular synthesis as well as in high-throughput screening methods (the "omics" revolution). Among the new crop of therapeutic agents in the market or under development, angiogenesis inhibitors, immunotherapeutic agents (monoclonal antibodies, cytokines and cancer vaccines), kinase signaling inhibitors and gene (DNA or RNA based) therapy seem to be the most promising. RNA (mRNA and RNAi) based approaches have been attracting considerable interest the last few years, as aberrant RNA regulation has been increasingly implicated in a range of cellular pathways and processes critical to cancer development.

Therefore, the market for targeted RNA-based therapies is still in its infancy and at the very early stages of development. The recent breakthroughs in elucidating the crucial role for RNA in a host of cellular mechanisms have facilitated the emergence of novel approaches for engineering mRNA and RNAi sequences into clinically approved therapeutics. These technologies aim to take advantage of the body's own natural processes, to either promote expression of beneficial proteins or silence genes and eliminate specific proteins in cancer cells, leading to the development of safer cancer therapeutics with vastly improved specificity and efficacy. Depending on the type and location of the targeted cancer, the delivery of the therapeutic agents often requires the development of novel transport technologies such as nanocapsules, nanoparticles, liposomes and PEG-ylated vesicles in order to minimize toxicity to healthy tissues and maximize effective therapeutic concentrations at the tumor site.

Our disclosure will offer significant advantages as targeted RNA-based therapy for cancer:

- Targeted delivery remains a major challenge for RNA therapeutics. Our *in vivo* studies have shown that mesenchymal stem cells can selectively migrate to tumors (homing), thus, the EVmiR™ complexes incorporate the necessary membrane receptors that will allow for the targeted delivery of miRNA to the tumor site. The unique specificity of the approach translates into increased efficacy against tumors, by avoiding the targeting of healthy cells and minimizing potential side effects.

- A key obstacle to effective miRNA-based therapy is the requirement for stability and successful delivery into target tissues. Unlike many other drugs, miRNAs do not freely diffuse into cells; thus, miRNAs require special delivery approaches to achieve the desired effect. We have demonstrated that the EVmiR™ complexes can fuse with the target cancer cells and allow therapeutic miRNA to incorporate into recipient cells.

- EVs can deliver therapeutic doses of miRNAs that have been shown to affect multiple pathways that are involved in cancer development. Specific miRNAs have already been identified, which regulate gene expression in cancer cells both through reduction or deletion of oncogenic miRNA and through amplification or overexpression of tumor suppressing miRNA. Thus, compared to earlier approaches targeting single genes, the miRNA approach may present opportunities to target multiple molecules in cancer cells, and thus offer multiple avenues for tumor reduction with a single approach.
- Unlike earlier RNA therapies, the EVmiR™ complexes do not elicit an innate immune response since the therapeutic miRNA sequences are not exposed. Instead, these EVs have been shown to induce immunosuppression (through cytokine inhibition), which renders the targeted delivery approach more effective, especially in recurring cases.

This disclosure represents a new clinical cancer therapeutic modality, with potential for significant commercialization and applications in future clinical investigations into the treatment of malignancies. This disclosure will lead to the rapid development of marketable products and services for patients and the transformation of the cancer treatment field by creating new knowledge on the pathology of tumors and allowing for the customization and personalization of treatments.

DETAILED DESCRIPTION

Collectively, 22 miRNAs are used in this embodiment in order to reduce tumor growth and metastasis in LCP-1 positive cancers. Stem cell-derived miRNAs (9 miRs naturally occurring) are assigned to be always present in the therapeutic cocktail responsible for targeting of cancer cells and reduction of the growth, invasion and proliferation of tumors. On the other hand, 13 miRNAs (synthetically or *de novo* induced) maybe used either selectively or in total depending on the diagnostic profile of selected patients. The miRNA cocktail will be mainly administered by means of the EVmiR™ complex but not limited to this formulation. Examples for the production and use of the technology are given below:

EV production: The present embodiment provides a method of treating cancers that are positive for LCP-1, as well as for any of the other oncoproteins from Table 1 (Protein Panel), with the EVmiR™ complex containing:

a) specific miRNAs that are able to downregulate the targeted oncoproteins (given in Table 1)

b) specific cell membrane proteins that enable EVs to target and home to sites of tumor growth and metastasis (given in Table 2)