Technical field

The present invention pertains to extracellular vesicle (EV) therapeutics, wherein the EVs are coated with proteins comprising Fc domains (such as antibodies) for i.a. targeting and therapeutic applications.

Background art

Protein biologics are routinely used in the treatment and/or prevention of a wide range of diseases, including cancer, genetic disorders and autoimmune diseases. Antibodies and chimeric receptors, among which many of today's blockbuster drugs can be found, are typically administered in naked form, i.e. without any delivery vehicles. Extracellular vesicles (EVs) are nano-sized vesicles that are released by EV-producing cells into the extracellular environment. EVs and in particular exosomes have been shown to be able to transport protein biologics, such as antibodies and decoy receptors, into target cells, enabling an entirely novel form of advanced biological therapeutics harnessing the properties of EVs in combination with the specificity of recombinant proteins.

The use of EVs to deliver protein therapeutics provides a number of advantages over conventional direct administration of biologics. For example, when biotherapeutics are delivered using EVs they are protected from degradation and are more stable; EVs constitute a multivalent drug delivery modality which may lead to enhanced efficacy; EVs may improve the pharmacokinetics and the pharmacodynamics of a protein biologic; EVs can be targeted to tissues and cells of interest; EVs may have inherent therapeutic effects reflecting their cellular origin; and, EVs also enable penetration of the blood-brain-barrier and improved CNS delivery.

Despite all their advantages, loading of large and complex protein biologics into EVs for subsequent delivery to a target cell has not proved entirely straightforward. WO2013/084000 and WO2014/168548 both describe successful loading of protein-based biologics (such as antibodies and decoy receptors) into and onto EVs. WO2013/084000 discloses both so called endogenous and exogenous loading of for instance antibodies into EVs such as exosomes. Exogenous loading refers to loading of EVs via introduction of a protein cargo molecule directly into EVs after their isolation from an EV-producing cell in culture. Exogenous introduction of a protein may be carried out, as in WO2013/084000, using for instance electroporation or transfection of the polypeptide of interest post isolation from the parental cell. Endogenous loading on the other hand involves, as taught for instance by WO2014/168548, transfecting an EV-producing cell with a polynucleotide construct which encodes the therapeutic protein of interest. WO2015/058148 teaches an example of endogenous loading of proteins of interest, namely genetic engineering of NK cells with construct encoding for Fc receptors such as CD64, CD32 and CD16. However, genetic modification of e.g. NK cells to express Fc receptors is not a particularly effective way of loading proteins of interest, as it will only result in a low number of Fc receptors per EV. Thus, the approach taught by WO2015/058148 does not at all address the challenges associated with efficient loading of EVs with multiple large and complex protein biologics, especially in a controllable, predictable, scalable, and cost-efficient manner.

Summary of the invention

It is hence an object of the present invention to overcome the above-identified problems associated with the loading of EVs with protein biologics, and especially loading of antibodies and other proteins comprising Fc domains, for subsequent therapeutic application. Furthermore, the present invention aims to satisfy other existing needs within the art, for instance to enable high-affinity and high-density coating of EVs with a substantial plurality of therapeutic, targeting, or anti-

clearance antibodies and other proteins comprising Fc domains (either naturally or as a result of protein engineering), to considerably enhance the therapeutic potential of EVs for therapeutic protein delivery.

The present invention achieves these and other objectives by utilizing fusion constructs comprising exosomal proteins fused to Fc binding polypeptides (herein often referred to as Fc binders) of human and/or non-human origin. The use of exosomal proteins as modalities for loading of EVs enables displaying a large number of Fc binders on the surface of EVs, which is important in order to be able to densely coat the EVs with proteins comprising Fc domains (herein often referred to as Fc containing proteins), which are bound to the EV via interaction between the Fc binders and the proteins comprising Fc domains (such Fc containing proteins may in advantageous embodiments be antibodies). Fc domains of human and/or mammalian origin that may be fused onto proteins natively lacking Fc domains may be selected from the following non-limiting list of alternatives: human IGHM (as a non-limiting example the accession number P01871), human IGHA1 (as a non-limiting example the accession number P01876), human IGHA2 (as a non-limiting example the accession number P01877), human IGKC (as a non-limiting example the accession number P01834), human IGHG1 (as a non-limiting example the accession number P01857), human IGHG2 (as a non-limiting example the accession number P01859), human IGHG3 (as a non-limiting example the accession number P01860), human IGHG4 (as a non-limiting example the accession number P01861), human IGHD (as a non-limiting example the accession number P01880), human IGHE (as a non-limiting example the accession number P01854), and any domains, derivatives, or combinations thereof. The use of non-human Fc binding polypeptides such as Protein A/G and the so called Z domain and the dimeric ZZ domain may result in higher binding affinity between the Fc binder and its interaction partner(s), i.e. the Fc domains of an Fc containing protein. Furthermore, non-human Fc binders are often smaller in size than human Fc binding proteins. On the other hand, Fc binding proteins and polypeptide domains of human and/or mammal origin, such as human FCGRI

(CD64) (as a non-limiting example the SEQ ID NO 31), FCGR2A (CD32A) (as a non-limiting example the accession number P12318), FCGR2B (CD32B) (as a non-limiting example the accession number P31994), FCGR2C (CD32C) (as a non-limiting example the accession number P31995), FCGR3A (CD16A) (as a non-limiting example the accession number P0837), FCGR3B (CD16B) (as a non-limiting example the accession number O75015), FCAMR (as a non-limiting example the SEQ ID NO 28), FCERA (as a non-limiting example the SEQ ID NO 30), FCAR (as a non-limiting example the SEQ ID NO 29), or mouse FCGRI (as a non-limiting example the SEQ ID NO 79), FCGRIIB (as a non-limiting example the SEQ ID NO 80), FCGRIII (as a non-limiting example the SEQ ID NO 81), FCGRIV (as a non-limiting example the SEQ ID NO 82), FCGRn (as a non-limiting example the SEQ ID NO 83), may offer advantages as they are mammal proteins and as such may be less immunostimulatory. Regardless, it is key to engineer the EVs so as to ensure that they comprise fusion constructs of the Fc binding polypeptides as opposed to merely overexpressed proteins that bind to Fc domains, in order to ensure adequate numbers of Fc binding polypeptides being displayed on EVs and to enable controllable production.

Furthermore, the Fc binder polypeptides of the present invention may be engineered to be smaller than natively expressed Fc binding proteins, which makes it easier to direct them to EV surfaces with the aid of fusion constructs with EV proteins. Moreover, one significant difference between non-human Fc binders (which may be e.g. bacterial in origin) and human Fc binders is the fact that such non-human Fc binders can in certain instances simultaneously bind more than one Fc domain, which may lead to increased surface coating of the EVs with the protein of interest comprising an Fc domain. For instance, Protein A/G, which is a fusion protein between Protein A derived from Staphylococcus aureus and from Streptococcus dysgalactiae, has seven binding regions for the Fc domain of IgG antibodies. This multivalency can lead to multiple proteins comprising Fc domains, such as antibodies, being bound to each Fc binder (in this case Protein A/G), enabling denser coating of the EVs with Fc domain-containing proteins, such as

antibodies. The denser coating of the EVs with the Fc binders importantly may also enable e.g. a higher avidity between e.g. antibodies and their corresponding antigens, meaning that the binding to the target of interest will be enhanced, which may be beneficial from a targeting and/or therapeutic standpoint.

In one aspect, the present invention relates to EVs comprising fusion proteins, wherein the fusion proteins comprise at least one polypeptide-based Fc binder fused to an exosomal polypeptide. As a result of the fusion with the EV protein, the Fc binders are efficiently displayed in high numbers on the surface of EVs, enabling dense coating of EVs with Fc domain-containing proteins such as antibodies. Coating of EVs with antibodies and other proteins comprising Fc domains (naturally or as a result of molecular biology engineering) is advantageous for several reasons: (1) antibodies or other proteins targeting a specific cell types, tissue, and/or organs represent a highly useful approach to re-directing distribution and optimizing delivery of EV-based therapeutics, (2) therapeutic antibodies or other Fc domain-containing proteins that interact with a target antigen of interest can be efficiently delivered to tissues of interest using EVs (for instance to the CNS or to the brain), (3) multiplexed antibodies or other Fc domain-containing proteins on the surface of EVs may be significantly better at binding targets, such as target antigens, (4) EVs are an advantageous modality for delivery of antibody-drug conjugates (ADCs) or receptor-drug conjugates, as multiplexing of ADCs may significantly enhance their therapeutic efficacy and their presence on EVs means they can also enter target cells, (5) EVs comprising Fc binders facilitate cellular internalization of Fc domain-containing proteins, such as antibodies, ADCs or essentially any protein comprising an Fc domain, either naturally or as a result of engineering, and (6) coating of EVs with antibodies or Fc domain-containing proteins may reduce opsonization and/or immune-mediated clearance of EVs, which may in turn be important for their therapeutic activity.

In another aspect, the present invention relates to complexes between fusion proteins as per the present invention and Fc domain-containing proteins (such as

antibodies and virtually any biopharmaceutical to which an Fc domain can be fused, e.g. an intracellularly active enzyme such as NPC1 or the nuclease Cas9). As above-mentioned the fusion protein comprises an Fc-binding polypeptide fused to an exosomal polypeptide, and the Fc binder binds to the Fc domain of the Fc domain-containing protein in the complex, which Fc domain-containing protein may be any protein of interest, for instance an antibody or any other protein comprising an Fc domain, either naturally or as a result of engineering of the protein in question. As a result of the EV trafficking capabilities of EV proteins, such non-covalent complexes between fusion proteins and Fc domain-containing proteins are typically present, e.g. anchored in the membrane of EV, resulting in an EV coated with a plurality of Fc domain-containing proteins which are capable of exerting their biological effects. The complexes may additionally or alternatively reside inside an EV.

In a further aspect, the present invention pertains to pharmaceutical compositions comprising EVs and/or non-covalent complexes, such as nanoparticle complexes (i.e. EVs decorated inside, outside, and/or in the EV membrane with a plurality of at least one type of Fc containing proteins) as per the present invention, and a pharmaceutically acceptable carrier. In further aspects, the present invention thus also relates to EVs, EV-protein complexes, and/or pharmaceutical compositions comprising such EVs and EV-protein complexes for use in medicine, preferably in the treatment of diseases which would benefit from antibody- or Fc domain-containing protein-based treatment, ADC-based treatment, and/or antibody-mediated targeting.

In further aspects, the present invention pertains to methods for producing EVs capable of binding to proteins comprising an Fc domain. Such methods may comprise the steps of: (i) introducing into an EV source cell a polynucleotide construct encoding a fusion protein comprising at least one Fc binder polypeptide and at least one exosomal polypeptide, and (ii) harvesting EVs which are secreted from the EV source cell, said EVs comprising the fusion protein of interest.

Additionally, the present invention relates to methods for coating EVs with at least one protein comprising an Fc domain, comprising the steps of (i) providing an EV comprising a fusion protein comprising at least one Fc binder and at least one exosomal polypeptide, and (ii) allowing the Fc binder of the fusion protein to bind the Fc domain of at least one protein comprising an Fc domain.

Finally, the present invention also relates to fusion proteins comprising at least one Fc binder and at least one exosomal polypeptide, and polynucleotide constructs encoding for such fusion proteins, as well as vectors, EVs and cells comprising such constructs.

Brief description of the figures

Figure 1. Schematic illustration of an EV comprising a fusion protein comprising an exosomal protein fused to an Fc binding polypeptide (i.e. the Fc binder domain). The Fc binder is capable of binding e.g. an antibody and/or any other protein comprising an Fc domain, thereby turning the EV into a multivalent delivery vehicle for protein therapeutics.

Figure 2. Electron microscopy pictures of EVs comprising Fc binding polypeptides (A) are decorated with nanogold labeled antibodies (i.e. Fc containing proteins), whereas control EVs (B), which lack Fc binding polypeptides, do not have any antibodies bound to their surfaces.

Figure 3. Flow cytometry data showing that the EVs comprising Fc binding polypeptides bind Fc containing proteins of interest (human IgG). The binding is very efficient to all bead populations included in the kit, including the unspecific/Isotype/negative control bead populations

Figure 4. Anti-HER2 antibody increases uptake of antibody-decorated EVs as compared to isotype control-decorated and wild type EVs only in HER2 high-

expressing cell line MDA-MB-361, but not in HER2 low-expressing cell line MDA-MB-231.

Figure 5. Etanercept-coated EVs protects mice from loss of body weight, as opposed to WT or control decorated-EVs, and displayed higher activity than naked etanercept, possible due to higher affinity between etanercept and TNFalpha when etanercept is multiplexed and/or when the Fc binding polypeptide binds to its Fc domain.

Figure 6. Signals of fluorescently labelled antibodies are clearly present in cells treated with Fc-binding EVs having attached to their surfaces antibodies comprising Fc domains, while fluorescence signals are absent in untreated (1) or control EV treated (2) cells, measured by fluorescence microscopy (A) and flow cytometry (B). This demonstrates that Fc containing proteins such as antibodies can be delivered intracellularly by Fc binding EVs, and that binding to EVs dramatically increases uptake of antibodies into cells.

Figure 7. Successful inhibition of NFkB-mediated intracellular signals when an anti-NFkB antibody is delivered into cells by Fc-binding EVs (i.e. EVs comprising at least one Fc binding polypeptide fused to at least one exosomal polypeptide).

Figure 8. Control EVs, as well as single treatment with an anti-integrin-4a antibody display moderate protective effect from EAE development in mice, whereas zz domain EVs coated with the same anti-integrin-4a antibody almost completely abrogates EAE development *in vivo*.

Figure 9. Intracellular delivery of CRISPR-Cas9 guide RNA complexes for targeted genome editing, graph showing targeted genomic cleavage after delivery of Fc Cas9 guide RNA complexes through Fc binding domain-Lamp2b engineered EVs.

Figure 10. Intracellular delivery of the lysosomal storage disorder enzyme GBA fused to an Fc domain, and bound to HEK-derived EVs comprising an Fc binding polypeptide.

Figure 11. Cyclin D levels following U2OS cell treatment with siRNA-loaded Ago2 attached to the surface of Lamp2b-ZZ domain EVs, resulting in significant target silencing.

Detailed description of the invention

The present invention relates to various aspects and embodiments pertaining to EVs comprising fusion proteins comprising at least one Fc binder fused to an exosomal polypeptide, to enable dense coating of EVs with antibodies and other Fc domain-containing proteins that can be sequestered by the Fc binder and used for therapeutic application in the treatment of various diseases and disorders.

For convenience and clarity, certain terms employed herein are collected and described below. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

Where features, aspects, embodiments, or alternatives of the present invention are described in terms of Markush groups, a person skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group. The person skilled in the art will further recognize that the invention is also thereby described in terms of any combination of individual members or subgroups of members of Markush groups. Additionally, it should be noted that embodiments and features described in connection with one of the aspects and/or embodiments of the present invention also apply mutatis mutandis to all the other aspects and/or embodiments of the invention. For example, the Fc binding polypeptides described herein in connection

with the EVs comprising fusion proteins comprising such Fc binding polypeptides are to be understood to be disclosed, relevant, and compatible with all other aspects, teachings and embodiments herein, for instance aspects and/or embodiments relating to the methods for producing or coating the EVs, or relating to the polynucleotide and polypeptide constructs described herein. Furthermore, certain embodiments described in connection with certain aspects, for instance the administration routes of the EVs comprising the fusion protein comprising the Fc binding polypeptide and the exosome polypeptide, as described in relation to aspects pertaining to treating certain medical indications, may naturally also be relevant in connection with other aspects and/or embodiment such as those pertaining to the pharmaceutical compositions and/or the Fc binding polypeptide – Fc containing protein complexes of the present invention. Furthermore, all polypeptides and proteins identified herein can be freely combined in fusion proteins using conventional strategies for fusing polypeptides. As a non-limiting example, all Fc binding polypeptides described herein may be freely combined in any combination with one or more exosomal polypeptides. Also, Fc binding polypeptides may be combined with each other to generate constructs comprising more than one Fc binding polypeptide. Moreover, any and all features (for instance any and all members of a Markush group) can be freely combined with any and all other features (for instance any and all members of any other Markush group), e.g. any Fc binding protein may be combined with any Fc containing protein such as any antibody, or any exosomal polypeptide may be combined with any Fc binding polypeptide. Furthermore, when teachings herein refer to EVs (and/or the EV-anchored fusion protein – Fc containing protein complexes) in singular and/or to EVs as discrete natural nanoparticle-like vesicles it should be understood that all such teachings are equally relevant for and applicable to a plurality of EVs and populations of EVs and the EVs coated with Fc containing proteins. As a general remark, the Fc binding polypeptides, the Fc containing proteins such as the antibodies, the EV-producing cell sources, the exosomal proteins, and all other aspects, embodiments, and alternatives in accordance with the present invention may be freely combined in any and all possible combinations without deviating

from the scope and the gist of the invention. Furthermore, any polypeptide or polynucleotide or any polypeptide or polynucleotide sequences (amino acid sequences or nucleotide sequences, respectively) of the present invention may deviate considerably from the original polypeptides, polynucleotides and sequences as long as any given molecule retains the ability to carry out the desired technical effect associated therewith. As long as their biological properties are maintained the polypeptide and/or polynucleotide sequences according to the present application may deviate with as much as 50% (calculated using for instance BLAST or ClustalW) as compared to the native sequence, although a sequence identity that is as high as possible is preferable (for instance 60%, 70%, 80%, or e.g. 90% or higher). The combination (fusion) of e.g. at least one Fc binding polypeptide and at least one exosomal protein implies that certain segments of the respective polypeptides may be replaced and/or modified and/or that the sequences may be interrupted by insertion of other amino acid stretches, meaning that the deviation from the native sequence may be considerable as long as the key properties (e.g. Fc binding properties, trafficking to the surface of exosomes, therapeutic activity, etc.) are conserved. Similar reasoning thus naturally applies to the polynucleotide sequences encoding for such polypeptides. All accession numbers and SEQ ID NOs mentioned herein in connection with peptides, polypeptides and proteins shall only be seen as examples and for information only, and all peptides, polypeptides and proteins shall be given their ordinary meaning as the skilled person would understand them. Thus, as above-mentioned, the skilled person will also understand that the present invention encompasses not merely the specific SEQ ID NOs and/or accession numbers referred to herein but also variants and derivatives thereof. All accession numbers referred to herein are UniProtKB accession numbers as per the 22 June 2017 version of the database, and all proteins, polypeptides, peptides, nucleotides and polynucleotides mentioned herein are to be construed according to their conventional meaning as understood by a skilled person.

The terms “extracellular vesicle” or “EV” or “exosome” are used interchangeably herein and shall be understood to relate to any type of vesicle that is obtainable from a cell in any form, for instance a microvesicle (e.g. any vesicle shed from the plasma membrane of a cell), an exosome (e.g. any vesicle derived from the endo-lysosomal pathway), an apoptotic body (e.g. obtainable from apoptotic cells), a microparticle (which may be derived from e.g. platelets), an ectosome (derivable from e.g. neutrophils and monocytes in serum), prostatosome (e.g. obtainable from prostate cancer cells), or a cardiosome (e.g. derivable from cardiac cells), etc. The sizes of EVs may vary considerably but an EV typically has a nano-sized hydrodynamic radius, i.e. a radius below 1000 nm. Clearly, EVs may be derived from any cell type, both in vivo, ex vivo, and in vitro. Furthermore, the said terms shall also be understood to relate to extracellular vesicle mimics, cell membrane-based vesicles obtained through for instance membrane extrusion, sonication, or other techniques, etc. It will be clear to the skilled artisan that when describing medical and scientific uses and applications of the EVs, the present invention normally relates to a plurality of EVs, i.e. a population of EVs which may comprise thousands, millions, billions or even trillions of EVs. As can be seen from the experimental section below, EVs may be present in concentrations such as $10^{5,}$ 10^{8}, 10^{10}, 10^{11}, 10^{12}, 10^{13}, 10^{14}, 10^{15}, 10^{18}, 10^{25} ,10^{30} EVs (often termed “particles”) per unit of volume (for instance per ml), or any other number larger, smaller or anywhere in between. In the same vein, the term “population”, which may e.g. relate to an EV comprising a certain fusion protein between an exosomal polypeptide and an Fc binding polypeptide which in turn may be bound to an Fc containing protein of interest, shall be understood to encompass a plurality of entities constituting such a population. In other words, individual EVs when present in a plurality constitute an EV population. Thus, naturally, the present invention pertains both to individual EVs and populations comprising EVs, as will be clear to the skilled person. The dosages of EVs when applied *in vivo* may naturally vary considerably depending on the disease to be treated, the administration route, the Fc containing protein of interest, any targeting moieties present on the EVs, the pharmaceutical formulation, etc. Furthermore, the EVs of the present invention

may also comprise additional therapeutic agents, in addition to the Fc containing proteins which may be bound to the EV surfaces. In some embodiments, the additional therapeutic agent may be at least one therapeutic small molecule drug. In some embodiments, the therapeutic small molecule drug may be selected from the group consisting of DNA damaging agents, agents that inhibit DNA synthesis, microtubule and tubulin binding agents, anti-metabolites, inducers of oxidative damage, anti-angiogenics, endocrine therapies, anti-estrogens, immuno-modulators such as Toll-like receptor agonists or antagonists, histone deacetylase inhibitors, inhibitors of signal transduction such as inhibitors of kinases, inhibitors of heat shock proteins, retinoids, inhibitors of growth factor receptors, anti-mitotic compounds, anti-inflammatories, cell cycle regulators, transcription factor inhibitors, and apoptosis inducers, and any combination thereof. In further embodiments, the additional therapeutic agent may be a therapeutic nucleic acid-based agent. Such nucleic acid-based therapeutic agents may be selected from the group comprising single-stranded RNA or DNA, double-stranded RNA or DNA, oligonucleotides such as siRNA, splice-switching RNA, CRISPR guide strands, short hairpin RNA (shRNA), miRNA, antisense oligonucleotides, polynucleotides such as mRNA, plasmids, or any other RNA or DNA vector. Of particular interest are nucleic acid-based agents which are chemically synthesized and/or which comprise chemically modified nucleotides such as 2'-O-Me, 2'-O-Allyl, 2'-O-MOE, 2'-F, 2'-CE, 2'-EA 2'-FANA, LNA, CLNA, ENA, PNA, phosphorothioates, tricyclo-DNA, etc. In yet further embodiments, the EVs as per the present invention may comprise additional therapeutic agents which may be protein and/or peptides. Such proteins and/or peptides may be present inside of the EVs, inserted into the EV membrane or in association with the EV membrane, or may be protruding from the EV into the extravesicular environment. Such therapeutic protein and/or peptide agents may be selected from a group of non-limiting examples including: antibodies, intrabodies, single chain variable fragments (scFv), affibodies, bi- and multispecific antibodies or binders, affibodies, darpins, receptors, ligands, enzymes for e.g. enzyme replacement therapy or gene editing, tumor suppressors, viral or bacterial inhibitors, cell component proteins, DNA and/or RNA binding

proteins, DNA repair inhibitors, nucleases, proteinases, integrases, transcription factors, growth factors, apoptosis inhibitors and inducers, toxins (for instance pseudomonas exotoxins), structural proteins, neurotrophic factors such as NT3/4, brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) and its individual subunits such as the 2.5S beta subunit, ion channels, membrane transporters, proteostasis factors, proteins involved in cellular signaling, translation- and transcription related proteins, nucleotide binding proteins, protein binding proteins, lipid binding proteins, glycosaminoglycans (GAGs) and GAG-binding proteins, metabolic proteins, cellular stress regulating proteins, inflammation and immune system regulating proteins, mitochondrial proteins, and heat shock proteins, etc. In a preferred embodiment, the therapeutic agent may be a CRISPR-associated (Cas) polypeptide (such as Cas9 (as a non-limiting example the accession number Q99ZW2)) with intact nuclease activity which is associated with (i.e. carries with it) an RNA strand that enables the Cas polypeptide to carry out its nuclease activity in a target cell once delivered by the EV. Alternatively, in another preferred embodiment, the Cas polypeptide may be catalytically inactive, to enable targeted genetic engineering. Yet another alternative may be any other type of CRISPR effector such as the single RNA-guided endonuclease Cpf1 (from species such as Acidaminococcus or Lachnospiraceae) (as non-limiting examples the accession numbers U2UMQ6 and A0Q7Q2). Additional preferred embodiments include therapeutic proteins selected from the group comprising enzymes for lysosomal storage disorders, for instance glucocerebrosidases such as imiglucerase, alpha-galactosidase, alpha-L-iduronidase, iduronate-2-sulfatase and idursulfase, arylsulfatase, galsulfase, acid-alpha glucosidase, sphingomyelinase, galactocerebrosidase, galactosylceramidase, glucosylceramidase (as a non-limiting example the accession number P04062) ceramidase, alpha-N-acetylgalactosaminidase, beta-galactosidase, lysosomal acid lipase, acid sphingomyelinase, NPC1 (as a non-limiting example the accession number O15118), NPC2 (as a non-limiting example the accession number P61916), heparan sulfamidase, N-acetylglucosaminidase, heparan-α-glucosaminide-N-acetyltransferase, N-acetylglucosamine 6-sulfatase, galactose-

6-sulfate sulfatase, galactose-6-sulfate sulfatase, hyaluronidase, alpha-N-acetyl neuraminidase, GlcNAc phosphotransferase, mucolipin1, palmitoyl-protein thioesterase, tripeptidyl peptidase I, palmitoyl-protein thioesterase 1, tripeptidyl peptidase 1, battenin, linclin, alpha-D-mannosidase, beta-mannosidase, aspartylglucosaminidase, alpha-L-fucosidase, cystinosin, cathepsin K, sialin, LAMP2, and hexoaminidase. In other preferred embodiments, the therapeutic protein may be e.g. an intracellular protein that modifies inflammatory responses, for instance epigenetic proteins such as methylases and bromodomains, or an intracellular protein that modifies muscle function, e.g. transcription factors such as MyoD (as a non-limiting example the accession number P15172) or Myf5, proteins regulating muscle contractility e.g. myosin, actin, calcium/binding proteins such as troponin, or structural proteins such as dystrophin (as a non-limiting example the accession number P11532), mini dystrophin (as a non-limiting example the accession number P15172), utrophin, titin, nebulin, dystrophin-associated proteins such as dystrobrevin, syntrophin, syncoilin, desmin, sarcoglycan, dystroglycan, sarcospan, agrin, and/or fukutin. Importantly, all of the above-mentioned therapeutic proteins may be engineered to contain an Fc domain, in order to enable binding to the Fc binding polypeptide present on the EVs. Another non-limiting example is the fusion of an Fc domain onto the enzyme NPC1 for subsequent delivery into a target cell. Yet another non-limiting example which may be utilized to improve intracellular bioactivity of EV-delivered Fc containing proteins (for instance Fc-Cas9 or antibodies) is to fuse an Fc domain to an endosomal escape peptide or protein, such as HA2, cell-penetrating peptides (CPPs) such as the TAT peptide, transportan, peneratin, poly-lysine, or gp41, cholera toxin, Shiga toxin, saporin, diphtheria toxin peptides, etc. Displaying such endosomal escape domains on the surface of an EV may enhance both internalization into target cells and subsequent endosomal escape. An advantageous non-limiting example of how an Fc domain can be fused onto a protein of interest is the fusion of an Fc domain onto Cas9, Cpf1, non-cleaving Cas variants, or any other type of gene editing protein or ribonucleoprotein (RNP) for EV-mediated delivery into a target cell. In a preferred embodiment, an Fc domain

is fused either N-terminally or C-terminally to Cas9, which has been pre-loaded in vitro with the single guide RNA (sgRNA) strand (Cas pre-loaded with RNA forms a so called ribonucleoprotein (RNP) complex). The resulting Fc domain-containing RNP complex thus formed is then allowed to be bound by the Fc binding polypeptides of a suitable EV to attached them to the EV surface, followed by delivery into target cells. Creation of the RNP complex can be achieved in different ways and with different RNA components, such as conventional single guide RNA, a synthetic guide RNA comprising both the crRNA and the tracrRNA optionally combined with a hairpin loop, crRNA, tracrRNA, and various combinations thereof. Repair templates for homology-directed recombination or non-homologous end-joining or any other repair or replacement mechanism may also be included in a pre-formed RNP which can then be attached to EVs using the Fc domain – Fc binding polypeptide linkage.

The terms “antibody” and “mAb” and “Ab” as described herein is to be understood to include both antibodies in their entirety (i.e. whole antibodies) and any derivatives thereof with antigen-binding properties. Conventionally, an antibody refers to a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, or an antigen binding-portion thereof. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as V_H) and a heavy chain constant region. Each light chain is comprised of a light chain variable region (abbreviated herein as V_L) and a light chain constant region. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Importantly, for the purposes of the present invention an antibody of interest preferably has an Fc domain or a derivative thereof to which the Fc binding polypeptides of the present invention can bind, in order to enable coating of the EV surface. Antibodies of use in the invention may be monoclonal antibodies (mAbs) or polyclonal antibodies, preferably mAbs. Antibodies of particular utility in

the invention may be chimeric antibodies, CDR-grafted antibodies, nanobodies, human or humanised antibodies or any derivative thereof as long as it can be bound by the Fc binding polypeptide, which are typically comprised in the fusion proteins as per the present invention. The production of antibodies is outside of the scope of the present invention but typically both monoclonal and polyclonal antibodies are raised experimental non-human mammals such as goat, rabbit, llama, camelids, rat or mouse, but suitable antibodies may also be the result of other production methodologies, e.g. the standard somatic cell hybridization technique of Kohler and Milstein. Hybridoma production in e.g. the mouse is a very well-established procedure and can be achieved using techniques well known in the art. An antibody of use in the invention may be a human antibody, humanized antibody, and/or any type of chimeric antibody. The term "human antibody", as used herein, is intended to include antibodies having variable regions in which both the framework and CDR regions are derived from human germline immunoglobulin sequences. The human antibodies of use in the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis in vitro or by somatic mutation in vivo). The term "antibody derivatives" refers to any modified form of an antibody, e.g. an antibody having an amino acid sequence that is modified in any way, or a conjugate of the antibody and another agent or antibody, bispecific antibodies, multispecific antibodies, antibody domains, etc. The term "humanized antibody" refers to antibodies in which CDR sequences derived from another mammalian species, such as a mouse, camelid, llama, etc., have been grafted onto human framework sequences. Additional framework region modifications may be made within the human framework sequences. Antibodies in accordance with the present invention may include all isotypes and subtypes such as IgG (for instance IgG1, IgG2, IgG3, IgG4, IgG2a, IgG2d, and IgG2c), IgA, IgM, IgM, IgD, etc., and monomers, dimers, and oligomers thereof. Further, antibodies as per the present invention may have several functions when displayed on EVs: (1) antibodies may target specific cell types, tissues, and/or organs in order to re-direct distribution and optimize delivery of EV-based therapeutics, (2) therapeutic

antibodies that interact with a target antigen of interest can be efficiently delivered to tissues of interest using EVs (for instance to the CNS or to the brain), (3) multiplexed antibodies on the surface of EVs may be significantly better at binding target antigens, (4) antibody-drug conjugates (ADCs) may be multiplexed on EVs to significantly enhance their therapeutic efficacy, (5) antibodies bound by Fc binding polypeptide may have higher affinity for the target, (6) antibodies coated onto EVs as per the present invention may be delivered intracellularly, and (7) coating of EVs with antibodies may reduce opsonization and/or immune-mediated clearance of EVs, which may in turn be important for therapeutic activity.

The terms “Fc containing protein” and “protein comprising an Fc domain” and “Fc domain-containing protein” and “Fc domain containing protein” and “Fc domain protein” and similar terms are used interchangeably herein and shall be understood to relate to any protein, polypeptide, or peptide (i.e. any molecule comprising a sequence of amino acids) which comprises at least one Fc domain, either naturally or as a result of engineering of the protein in question to introduce an Fc domain. Fc stands for “fragment crystallizable”, which is the name of the tail regions of antibodies. Fc domains can however also be created and used on other proteins, not only antibodies. Non-limiting examples of such Fc domain-containing proteins include antibodies and antibody derivatives, Fc-modified decoy receptors and/or signal transducers such as interleukin decoy receptors for IL1, IL2, IL3, IL4, IL5, IL6 (such as the signal transducer gp130 (as a non-limiting example the accession number P40189)), IL7, IL8, IL9, IL10, IL11, IL12, IL13, IL14, IL15, IL17 (such as IL17R, with as a non-limiting example the accession number Q96F46), IL23 (such as IL23R, with as a non-limiting example the accession number Q5VWK5), etc., Fc domain-containing bi- and multi-specific binders, any type of Fc domain-containing receptors or ligands, Fc domain-modified enzymes for e.g. enzyme replacement therapy or gene editing, nucleases such as Cas and Cas9 onto which an Fc domain has been grafted, tumor suppressors fused to Fc domains, etc. Suitable Fc domains that may be fused with a protein of interest natively lacking an Fc domain include the following non-limiting examples: human

IGHM (as a non-limiting example the accession number P01871), human IGHA1 (as a non-limiting example the accession number P01876), human IGHA2 (as a non-limiting example the accession number P01877), human IGKC (as a non-limiting example the accession number P01834), human IGHG1 (as a non-limiting example the accession number P01857), human IGHG2 (as a non-limiting example the accession number P01859), human IGHG3 (as a non-limiting example the accession number P01860), human IGHG4 (as a non-limiting example the accession number P01861), human IGHD (as a non-limiting example the accession number P01880), human IGHE (as a non-limiting example the accession number P01854), and any domains, derivatives, or combinations thereof. In essence, any protein of interest may be modified to incorporate an Fc domain. Non-limiting examples of proteins onto which an Fc domain can be introduced include for instance tumor suppressors, viral or bacterial inhibitors, cell component proteins, DNA and/or RNA binding proteins, DNA repair inhibitors, nucleases, proteinases, integrases, transcription factors, growth factors, apoptosis inhibitors and inducers, toxins (for instance pseudomonas exotoxins), structural proteins, neurotrophic factors such as NT3/4, brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) and its individual subunits such as the 2.5S beta subunit, ion channels, membrane transporters, proteostasis factors, proteins involved in cellular signaling, translation- and transcription related proteins, nucleotide binding proteins, protein binding proteins, lipid binding proteins, glycosaminoglycans (GAGs) and GAG-binding proteins, metabolic proteins, cellular stress regulating proteins, inflammation and immune system regulating proteins, mitochondrial proteins, and heat shock proteins, etc. The above list of proteins of interest is not exhaustive and other proteins may also be relevant, as long as the protein either comprises an Fc domain or as long as it is possible to engineer the protein in question to comprise an Fc domain. One non-limiting example of such engineering of a protein to introduce an Fc domain includes adding an Fc domain to a decoy receptor, e.g. adding an Fc domain onto the Cas or Cas9 enzymes for bioactive delivery into target cells to enable gene editing. Another non-limiting example of such engineering of a protein to introduce an Fc

domain includes adding an Fc domain onto an enzyme for enzyme replacement therapy (for instance Fc domain-glucocerebrosidase or Fc domain-α-galactosidase or Fc domain-NPC1). A well-known example of a commercially available Fc domain-modified protein is etanercept, which is a biopharmaceutical for the treatment of various autoimmune disease, comprising the Fc domain of IgG fused onto TNF receptor 2. Thus, as is clear from the above, Fc domain-containing proteins as per the present invention may be essentially any protein of interest that contains an Fc domain, either naturally or as a result of introduction thereof.

The Fc containing proteins are often described herein as being "attached to" an EV and/or to an Fc binding polypeptide. Alternatively, EVs are sometimes referred to as being "coated by" Fc containing proteins, or as having "bound to their surface" or "attached to their surface" the Fc containing proteins. These terms shall be understood in the context of the conventional interaction between an Fc binding polypeptide and an Fc domain, that is that the two polypeptides are interacting with each other in a way that results in a chemical bond (typically a non-covalent bond) forming between the Fc binder and the Fc domain. Thus, this normally means that the EV which comprises the Fc binding polypeptide therefore has attached to it, by virtue of the chemical bond, the Fc domain of the Fc containing protein. As will be understood by the skilled person, an EV may consequently have a plurality of such Fc containing proteins bound (attached) to it, resulting in a form of coating when the binding is taking place on the EV surface.

The terms "Fc binding polypeptide" and "Fc binding protein" and "Fc binder" and "Fc-binding protein" and "binder" are used interchangeably herein and shall be understood to relate to any protein, polypeptide, or peptide (i.e. any molecule comprising a sequence of amino acids) which can bind an Fc domain of any protein of interest. Typically, the Fc binding polypeptides of the present invention are derived from various sources that are either human or non-human (e.g. mammal sources, bacteria, etc.), they have high affinity for Fc domains of various antibody isotypes, subtypes, and species (for instance IgG (as non-limiting examples in the

case of IgG, IgG1, IgG2, IgG3, IgG4, IgG2a, IgG2d, and/or IgG2c), IgA, IgM, IgM, IgD, etc.), and they can be fused to EV proteins. Non-limiting examples of Fc binding polypeptides in accordance with the present invention include, in addition to other Fc binding polypeptides mentioned through the present application, Protein A (as a non-limiting example the SEQ ID NO 77), Protein G (as a non-limiting example the SEQ ID NO 78), Protein A/G (as a non-limiting example the SEQ ID NO 72), Z domain (as a non-limiting example the SEQ ID NO 73), ZZ domain (two operably linked copies of as a non-limiting example the SEQ ID NO 73, i.e. as a non-limiting example the SEQ ID NO 74), human FCGRI (as a non-limiting example the SEQ ID NO 31), human FCGRIIA (as a non-limiting example the SEQ ID NO 33), human FCGRIIB (as a non-limiting example the accession number 31994), human FCGRIIC (as a non-limiting example the accession number 31995), human FCGRIIIA (as a non-limiting example the accession number P08637), human FCGR3B (as a non-limiting example the accession number O75015), human FCAMR (as a non-limiting example the SEQ ID NO 28), human FCERA, human FCAR, mouse FCGRI (as a non-limiting example the SEQ ID NO 79), mouse FCGRIIB (as a non-limiting example the SEQ ID NO 80), mouse FCGRIII (as a non-limiting example the SEQ ID NO 81), mouse FCGRIV (as a non-limiting example the SEQ ID NO 82), mouse FCGRn (as a non-limiting example the SEQ ID NO 83), and various combinations, derivatives, or alternatives thereof.

The terms “EV protein” and “EV polypeptide” and “exosomal polypeptide” and “exosomal protein” are used interchangeably herein and shall be understood to relate to any polypeptide that can be utilized to transport a polypeptide construct (which typically comprises, in addition to the EV protein, an Fc binding polypeptide) to a suitable vesicular structure, i.e. to a suitable EV. More specifically, these terms shall be understood as comprising any polypeptide that enables transporting, trafficking or shuttling of a fusion protein construct to a vesicular structure, such as an EV. Examples of such exosomal polypeptides are for instance CD9 (as a non-limiting example the SEQ ID NO 1), CD53 (as a non-limiting example the SEQ ID

NO 2), CD63 (as a non-limiting example the SEQ ID NO 3), CD81 (as a non-limiting example the SEQ ID NO 4), CD54 (as a non-limiting example the SEQ ID NO 5), CD50 (as a non-limiting example the SEQ ID NO 6), FLOT1 (as a non-limiting example the SEQ ID NO 7), FLOT2 (as a non-limiting example the SEQ ID NO 8), CD49d (as a non-limiting example the SEQ ID NO 9), CD71 (also known as the transferrin receptor) (as a non-limiting example the SEQ ID NO 10) and its endosomal sorting domain, i.e. the transferrin receptor endosomal sorting domain (as a non-limiting example the SEQ ID NO 23), CD133 (as a non-limiting example the SEQ ID NO 11), CD138 (syndecan-1) (as a non-limiting example the SEQ ID NO 12), CD235a (as a non-limiting example the SEQ ID NO 13), ALIX (as a non-limiting example the SEQ ID NO 14), Syntenin-1 (as a non-limiting example the SEQ ID NO 15), Syntenin-2 (as a non-limiting example the SEQ ID NO 16), Lamp2b (as a non-limiting example the SEQ ID NO 17), syndecan-2 (as a non-limiting example the SEQ ID NO 20), syndecan-3 (as a non-limiting example the SEQ ID NO 21), syndecan-4 (as a non-limiting example the SEQ ID NO 22), TSPAN8, TSPAN14, CD37, CD82, CD151, CD231, CD102, NOTCH1, NOTCH2, NOTCH3, NOTCH4, DLL1, DLL4, JAG1, JAG2, CD49d/ITGA4, ITGB5, ITGB6, ITGB7, CD11a, CD11b, CD11c, CD18/ITGB2, CD41, CD49b, CD49c, CD49e, CD51, CD61, CD104, Fc receptors, interleukin receptors, immunoglobulins, MHC-I or MHC-II components, CD2, CD3 epsilon, CD3 zeta, CD13, CD18, CD19 (as a non-limiting example the SEQ ID NO 26), CD30 (as a non-limiting example the SEQ ID NO 27), TSG101, CD34, CD36, CD40, CD40L, CD44, CD45, CD45RA, CD47, CD86, CD110, CD111, CD115, CD117, CD125, CD135, CD184, CD200, CD279, CD273, CD274, CD362, COL6A1, AGRN, EGFR, GAPDH, GLUR2, GLUR3, HLA-DM, HSPG2, L1CAM, LAMB1, LAMC1, LFA-1, LGALS3BP, Mac-1 alpha, Mac-1 beta, MFGE8, SLIT2, STX3, TCRA, TCRB, TCRD, TCRG, VTI1A, VTI1B, other exosomal polypeptides, and any combinations thereof, but numerous other polypeptides capable of transporting a polypeptide construct to an EV are comprised within the scope of the present invention. Typically, in many embodiments of the present invention, at least one exosomal polypeptide is fused to at least one Fc binding polypeptide, in order to form a fusion protein present in

an EV. Such fusion proteins may also comprise various other components to optimize their function(s), including linkers, transmembrane domains, cytosolic domains, multimerization domains, etc.

The terms “source cell” or “EV source cell” or “parental cell” or “cell source” or “EV-producing cell” or any other similar terminology shall be understood to relate to any type of cell that is capable of producing EVs under suitable conditions, for instance in suspension culture or in adherent culture or any in other type of culturing system. Source cells as per the present invention may also include cells producing exosomes in vivo. The source cells per the present invention may be select from a wide range of cells and cell lines, for instance mesenchymal stem or stromal cells or fibroblasts (obtainable from e.g. bone marrow, adipose tissue, Wharton’s jelly, perinatal tissue, tooth buds, umbilical cord blood, skin tissue, etc.), amnion cells and more specifically amnion epithelial cells optionally expressing various early markers, myeloid suppressor cells, M2 polarized macrophages, adipocytes, endothelial cells, fibroblasts, etc. Cell lines of particular interest include human umbilical cord endothelial cells (HUVECs), human embryonic kidney (HEK) cells, endothelial cell lines such as microvascular or lymphatic endothelial cells, chondrocytes, MSCs of different origin, airway or alveolar epithelial cells, fibroblasts, endothelial cells, etc. Also, immune cells such as B cells, T cells, NK cells, macrophages, monocytes, dendritic cells (DCs) are also within the scope of the present invention, and essentially any type of cell which is capable of producing EVs is also encompassed herein. Generally, EVs may be derived from essentially any cell source, be it a primary cell source or an immortalized cell line. The EV source cells may be any embryonic, fetal, and adult somatic stem cell types, including induced pluripotent stem cells (iPSCs) and other stem cells derived by any method. When treating neurological diseases, one may contemplate to utilize as source cells e.g. primary neurons, astrocytes, oligodendrocytes, microglia, and neural progenitor cells. The source cell may be either allogeneic, autologous, or even xenogeneic in nature to the patient to be treated, i.e. the cells may be from the patient himself or from an unrelated, matched or unmatched donor. In certain

contexts, allogeneic cells may be preferable from a medical standpoint, as they could provide immuno-modulatory effects that may not be obtainable from autologous cells of a patient suffering from a certain indication. For instance, in the context of treating systemic, peripheral and/or neurological inflammation, allogeneic MSCs may be preferable as EVs obtainable from such cells may enable immuno-modulation via e.g. macrophage and/or neutrophil phenotypic switching (from pro-inflammatory M1 or N1 phenotypes to anti-inflammatory M2 or N2 phenotypes, respectively).

In a first aspect, the present invention relates to EVs comprising fusion proteins, wherein the fusion proteins comprise at least one Fc binding polypeptide fused to an exosomal polypeptide. As a result of the fusion with the EV protein, the Fc binding polypeptide are efficiently transported to and displayed in high numbers on the surface of EVs, which enables subsequent coating of EVs with a various types of Fc containing proteins, typically therapeutic proteins endowed with an Fc domain, targeting antibodies, therapeutic antibodies, antibody-drug conjugates, and/or antibodies that are passively bound *in vivo* or purposely selected to reduce opsonization and recognition by immune cells (to prolong the circulation time of the EVs).

Thus, in an embodiment, an EV according to the first aspect has bound to it a plurality of Fc containing proteins through interaction between the Fc binding polypeptide and the Fc domains of the plurality of Fc containing proteins, wherein the plurality of Fc containing proteins may be the same or different. The EVs may be coated with a plurality of proteins comprising an Fc domain through the non-covalent interaction between the Fc binder and the at least one protein comprising an Fc domain. Said plurality may be at least 10, at least 20 or at least 30 proteins comprising an Fc domain.

In one embodiment of the invention, the Fc binders are of non-human origin, they may be obtained e.g. from bacteria, viruses, or non-human mammals. In another

embodiment, the Fc binders are of human or mammal origin. In preferred embodiments, the at least one Fc binding polypeptide may be selected from the group comprising Protein A (as a non-limiting example the SEQ ID NO 77), Protein G (as a non-limiting example the SEQ ID NO 78), Protein A/G (as a non-limiting example the SEQ ID NO 72), Z domain (as a non-limiting example the SEQ ID NO 73), ZZ domain (as a non-limiting example the SEQ ID NO 74), Protein L (as a non-limiting example the pdb id no 1HEZ), Protein LG, human FCGRI (as a non-limiting example the SEQ ID NO 31), human FCGR2A (as a non-limiting example the accession number P12318), human FCGR2B (as a non-limiting example the accession number P31994), human FCGR2C (as a non-limiting example the accession number P31994), human FCGR3A (as a non-limiting example the accession number P08637), human FCGR3B (as a non-limiting example the accession number O75015), human FCGRB (as a non-limiting example the accession number Q92637) (as a non-limiting example the SEQ ID NO 32), human FCAMR (as a non-limiting example the SEQ ID NO 28), human FCERA (as a non-limiting example the SEQ ID NO 30), human FCAR (as a non-limiting example the SEQ ID NO 29), mouse FCGRI (as a non-limiting example the SEQ ID NO 79), mouse FCGRIIB (as a non-limiting example the SEQ ID NO 80), mouse FCGRIII (as a non-limiting example the SEQ ID NO 81), mouse FCGRIV (as a non-limiting example the SEQ ID NO 82), mouse FCGRn (as a non-limiting example the SEQ ID NO 83), and any combination of any of the above Fc binding polypeptides. Other suitable Fc binding polypeptides, which have been obtained from e.g. phage display screening and via bioinformatics, include the Fc binding peptides SPH (as a non-limiting example the SEQ ID NO 57), SPA (as a non-limiting example the SEQ ID NO 58), SPG2 (as a non-limiting example the SEQ ID NO 59), SpA mimic 1 (as a non-limiting example the SEQ ID NO 60), SpA mimic 2 (as a non-limiting example the SEQ ID NO 61), SpA mimic 3 (as a non-limiting example the SEQ ID NO 62), SpA mimic 4 (as a non-limiting example the SEQ ID NO 63), SpA mimic 5 (as a non-limiting example the SEQ ID NO 64), SpA mimic 6 (as a non-limiting example the SEQ ID NO 65), SpA mimic 7 (as a non-limiting example the SEQ ID NO 66), SpA mimic 8 (as a non-limiting example the SEQ ID NO 69), SpA mimic

9 (as a non-limiting example the SEQ ID NO 70), SpA mimic 10 (as a non-limiting example the SEQ ID NO 71), Fcγ mimic 1 (as a non-limiting example the SEQ ID NO 67), and Fcγ mimic 2 (as a non-limiting example the SEQ ID NO 68), and any combination or derivative thereof. The selection of the most suitable Fc binding polypeptide for a particular construct depends heavily on the desired binding characteristics, the affinity, the orientation of the Fc binding polypeptide when fused to an exosomal polypeptide, and various other factors.

Protein A/G is a recombinant genetically engineered protein comprised of 7 Fc-binding domains EDABC-C1C3, with the Protein A part being obtained from Staphylococcus aureus segments E, D, A, B and C, and the Protein G part from Streptococcus segments C1 and C3. Advantageously, Protein A/G (as a non-limiting example the SEQ ID NO 72) has a broader binding capacity than either Protein A (as a non-limiting example the SEQ ID NO 77) or Protein G (as a non-limiting example the SEQ ID NO 78) alone and it has a broad binding affinity for antibodies from various species. Protein A/G binds to various human, mouse and rat IgG subclasses such as the human IgG1, IgG2, IgG3, IgG4; mouse IgG2a, IgG2b, IgG3 and rat IgG2a, IgG2c. In addition, Protein A/G binds to total IgG from cow, goat, sheep, horse, rabbit, guinea pig, pig, dog and cat. Protein A/G has been engineered to remove the cell wall-binding region, the cell membrane-binding region and albumin-binding region to enable strong binding to the Fc domain of a protein of interest. Thus, in advantageous embodiments as per the present invention, the Fc binder comprises more than one Fc binding region, as is the case with Protein A, Protein G, and Protein A/G. In an alternative embodiment, the Fc binder may be multiplied in order to enable binding to more than one copy of an antibody of interest. For instance, the short Z domain Fc binder may be included in the fusion protein in more than one copy, through an operational linkage allowing for binding to more than one Fc domain. This way it is possible to multiplex antibodies and other Fc domain-containing proteins not only between separate fusion proteins but also within one single fusion protein, which thus may bind more than one antibody. For instance, when Fc binding polypeptides are introduced into

EV proteins belonging to the tetraspanin family (such as CD63) it may be advantageous to insert one Fc binder on one loop and another Fc binder (which can be the same or different) on another loop of the protein. The Fc binder can be placed on inward-facing and/or outward-facing loops, depending on whether the Fc containing protein is meant to be loaded into the lumen of the EV or onto the surface of the EV. Some non-limiting examples of fusion proteins as per the present invention can be described schematically as follows (the below notation is not to be construed as illustrating any C and/or N terminal direction, it is merely meant for illustrative purposes):

Exosomal polypeptide – Fc binding polypeptide – Fc binding polypeptide

Exosomal polypeptide domain – Fc binding polypeptide – Exosomal polypeptide domain – Fc binding polypeptide

Exosomal polypeptide domain – Fc binding polypeptide A – Exosomal polypeptide domain – Fc binding polypeptide B

In some embodiments, the fusion proteins comprising the exosomal polypeptide and the Fc binding polypeptide may also contain additional polypeptides, polypeptide domains or sequences. Such additional polypeptide domains may exert various functions, for instance such domains may (i) contribute to enhancing the EV surface display of the fusion protein, (ii) lead to clustering of the fusion proteins thereby increasing the avidity of the Fc binding polypeptides, (iii) function as linkers to optimize the interaction between the exosomal polypeptides and the Fc binding polypeptide, and/or (iv) improve anchoring in the EV membrane, as well as various other functions. Two such additional polypeptides that may advantageously be included in part or as a whole in the fusion proteins of the preset invention are gp130 (as a non-limiting example the SEQ ID NO 18) and the tumor necrosis factor receptor 1 (TNFR1) (as a non-limiting example the SEQ ID NO 19). In particular, the transmembrane domains of these additional polypeptides may be

highly useful to optimize the insertion into the EV membrane and the display of the Fc binding polypeptide. Overall, various transmembrane domains may be highly advantageous as additional domains in the fusion proteins. For instance, when using the exosomal protein syntenin it is highly advantageous to insert an additional polypeptide domain, such the transmembrane domain of TNFR or the transmembrane domain of gp130, between syntenin and the Fc binding polypeptide of the fusion protein. Further additional domains may include multimerization domains such as fold-on domains, leucine zipper domains and/or trimerization domains, in order to increase surface display and/or avidity. A non-limiting schematic example of this is shown below:

Syntenin – cytosolicTNRF – Foldon trimerization domain - transmembraneTNFR domain – Z domain – extracellularTNFR domain

Syndecan – leuzine zipper domain - gp130cytosolic domain – gp130 transmembrane domaine – Fc binding polypeptide - gp130 extracellular domain

In a further embodiment, the exosomal polypeptide may be selected from the group comprising CD9, CD53, CD63, CD81, CD54, CD50, FLOT1, FLOT2, CD49d, CD71, CD133, CD138, CD235a, ALIX, Syntenin-1, Syntenin-2, Lamp2b, TSPAN8, syndecan-1, syndecan-2, syndecan-3, syndecan-4, TSPAN14, CD37, CD82 (as a non-limiting example the SEQ ID NO 24), CD151, CD231, CD102, NOTCH1, NOTCH2, NOTCH3, NOTCH4, DLL1, DLL4, JAG1, JAG2, CD49d/ITGA4, ITGB5, ITGB6, ITGB7, CD11a, CD11b, CD11c, CD18/ITGB2, CD41, CD49b, CD49c, CD49e, CD51, CD61, CD104, Fc receptors, interleukin receptors, immunoglobulins, MHC-I or MHC-II components, CD2, CD3 epsilon, CD3 zeta, CD13, CD18, CD19, CD30, CD34, CD36, CD40, CD40L, CD44, CD45, CD45RA, CD47, CD86, CD110, CD111, CD115, CD117, CD125, CD135, CD184, CD200, CD279, CD273, CD274, CD362, COL6A1, AGRN, EGFR, GAPDH, GLUR2, GLUR3, HLA-DM, HSPG2, L1CAM, LAMB1, LAMC1, LFA-1, LGALS3BP, Mac-1 alpha, Mac-1 beta, MFGE8 (as a non-limiting example the SEQ ID NO 25),

SLIT2, STX3, TCRA, TCRB, TCRD, TCRG, VTI1A, VTI1B, other exosomal polypeptides, and any combinations thereof.

Particularly advantageous fusion proteins as per the present invention may comprise the human exosomal proteins CD63, CD81, CD9, CD71 (transferrin receptor), Lamp2, and syntenin, fused to at least one copy of the following Fc binding polypeptides: Z domain, Protein A, Protein G, Protein A/G, FCGRI, human FCGR2A, human FCGR2B, human FCGR2C, human FCGR3A, human FCGR3B, human FCGR3C, human FCAMR, human FCAR, and human FCERA. Specifically, fusion of two Z domains to the EV protein CD63 (as a non-limiting example the SEQ ID NO 3) results in a highly potent Fc binder which is displayed in high quantities on the surface of EVs, as a consequence of the small size of the Z domain and the high EV surface expression of CD63. Display of the Fc binder on the outer surface of EVs is naturally preferable in order to enable exogenous binding of Fc domain-containing proteins of interest. In the case of CD63-ZZ domain fusion proteins, Z domains may be inserted into the first and second loop of CD63, to enable display on the outer EV surface after anchoring of CD63 into the EV membrane. Furthermore, as above-mentioned, suitable additional polypeptides and polypeptide domains and linkers may also be included in the fusion proteins, which normally comprises an exosomal polypeptide and an Fc binding polypeptide. Such additional polypeptides include domains from various cytokine receptors such as TNFR1 and TNFR2, the IL6 signal transducer gp130, and various other cytokines and cytokine-related polypeptides. In certain instances, cytokines and cytokine-related polypeptides (such as various cytokine receptors) may alone be able to transport Fc binding polypeptides to EVs. Linkers of particular utility are multiples and/or combinations of small and flexible amino acids such as glycine (G) and serine (S), typically denoted GS, for instance 2XGS or 4XGS. His tags for simplifying purification and assaying may also be added, both C terminally and N terminally, and so may various fluorescent proteins such as GFP. The following are particularly advantageous non-limiting examples of fusion proteins comprising at least one exosomal polypeptide and at least one Fc

binding polypeptide, often connected via linkers and optionally including an additional polypeptide(s) or polypeptide domains:

- CD81-Protein A/G CD81 Second loop (as a non-limiting example the SEQ ID NO 75)
- CD9-ZZ domain CD9 Second loop (as a non-limiting example the SEQ ID NO 76)
- FCAR extracellular domain-2XGGGgSlinker-Lamp2b (as a non-limiting example the SEQ ID NO 55)
- FCAR extracellular domain-4XGSlinker-Lamp2b (as a non-limiting example the SEQ ID NO 54)
- FCGR1A Extracellular domain-2XGGGgSlinker-Lamp2b (as a non-limiting example the SEQ ID NO 49)
- FCGR1A Extracellular domain-4XGSlinker-Lamp2b (as a non-limiting example the SEQ ID NO 48)
- FcRN Extracellular domain-4XGSlinker-Lamp2b (as a non-limiting example the SEQ ID NO 39)
- FcRN-2XGGGgSlinker-Lamp2b (as a non-limiting example the SEQ ID NO 40).
- Gp130 Extracellular domain-2XGGGGS linker-FCAR extracellular domain-Gp130 transmembrane domain-Leucine Zipper-N terminal syntenin (as a non-limiting example the SEQ ID NO 52)
- Gp130 Extracellular domain-2XGGGGS linker-FCGR1A Extracellular domain-Gp130 transmembrane domain-Leucine Zipper-N terminal syntenin (as a non-limiting example the SEQ ID NO 46)
- Gp130 Extracellular domain-2XGGGGS linker-FcRN Extracellular domain-Gp130 transmembrane domain-Leucine Zipper-N terminal syntenin (as a non-limiting example the SEQ ID NO 43)
- Gp130 Extracellular domain-2XGGGGS linker-Z domain-Gp130 transmembrane domain-Leucine Zipper-N terminal syntenin (as a non-limiting example the SEQ ID NO 34)

- Transferrin receptor-2XGGGGSlinker-FCAR extracellular domain (as a non-limiting example the SEQ ID NO 56)
- Transferrin receptor-2XGGGGSlinker-FCGR1A Extracellular domain (as a non-limiting example the SEQ ID NO 50)
- Transferrin receptor-2XGGGGSlinker-FcRN Extracellular domain (as a non-limiting example the SEQ ID NO 41)
- Transferrin receptor-2XGGGGSlinker-Z domain (as a non-limiting example the SEQ ID NO 38)
- CD63-FCAR extracellular domain CD63 First loop and CD63 Second loop (as a non-limiting example the SEQ ID NO 53)
- CD63-FCGR1A Extracellular domain CD63 First loop and CD63 Second loop (as a non-limiting example the SEQ ID NO 47)
- CD63-FcRN Extracellular domain CD63 First loop and CD63 Second loop (as a non-limiting example the SEQ ID NO 44)
- CD63-Z domain CD63 First loop and CD63 Second loop (as a non-limiting example the SEQ ID NO 35)
- TNFR Extracellular domain-2XGGGGS linker-FCAR extracellular domain-TNFR transmembrane domain-foldon-N terminal syntenin (as a non-limiting example the SEQ ID NO 51)
- TNFR Extracellular domain-2XGGGGS linker-FCGR1A Extracellular domain-TNFR transmembrane domain-foldon-N terminal syntenin (as a non-limiting example the SEQ ID NO 45)
- TNFR Extracellular domain-2XGGGGS linker-FcRN Extracellular domain-TNFR transmembrane domain-foldon-N terminal syntenin (as a non-limiting example the SEQ ID NO 42)
- TNFR Extracellular domain-2XGGGGS linker-Z domain-TNFR transmembrane domain-foldon-N terminal syntenin (as a non-limiting example the SEQ ID NO 33)
- Z domain-2XGGGgSlinker-Lamp2b (as a non-limiting example the SEQ ID NO 37)

- Z domain-4XGSlinker-Lamp2b (as a non-limiting example the SEQ ID NO 36)
- Transferrin receptor – Protein AG (as a non-limiting example the SEQ ID NO 10 operably fused to as a non-limiting example the SEQ ID NO 72)

The above-mentioned fusion proteins are merely examples of the many engineering possibilities the present invention allows for and as such they are merely non-limiting embodiments of the present invention. All components of the fusion proteins as per the present invention may be freely combined, e.g. the fusion proteins may contain one or several exosomal polypeptides which may be placed C terminally, N terminally, or both, or anywhere in the fusion protein. Further, the fusion proteins may also contain one or several Fc binding polypeptides, which may be placed C terminally, N terminally, or both, or on one or more of any loops of e.g. transmembrane parts, or anywhere in the fusion protein. For clarity, more than one type of exosomal polypeptide and more than one type of Fc binding polypeptide may be comprised in a single construct. Furthermore, additional stretches of amino acids such as linkers (often comprising the amino acids glycine and serine) and His tags may be included to simplify purification, assaying and visualization. Also, other peptides and polypeptide domains may also be included anywhere in the fusion protein sequence. For instance, various domains and regions from various cytokine receptors may advantageously be included, for instance various domains of TNFR1, TNFR2, IL17R, IL23R, gp130, IL6R, etc.

In a further embodiment, the Fc binding polypeptides may as above-mentioned bind to any protein comprising an Fc domain, not only antibodies but also other proteins comprising Fc domains, both naturally occurring and engineered Fc domain-containing proteins, such as the ones mentioned in several instances above. Advantageously, the present invention results in EVs coated with a plurality of proteins comprising an Fc domain, through interaction between the Fc binding polypeptide and at least one Fc containing protein. The interaction between the Fc binder and the Fc containing protein is normally based on non-covalent bonds

between the Fc binding polypeptide and the Fc domain of the Fc containing protein. Naturally, one single EV may be coated with more than one type of Fc domain-containing protein. In one non-limiting example, the Fc binding EVs are coated with one antibody targeting a suitable target along the PD1 axis, whereas another antibody is targeting a suitable target along the CTLA4 axis. In another non-limiting example, the Fc binding EVs are coated with an antibody targeting a tumor cell surface receptor and Cas9 fused to an Fc domain. One single EV may also, as is typically the case, comprise a substantial plurality of one single type of Fc domain-containing protein, such as one type of monoclonal antibody. Various combinations of targeting antibodies, therapeutic antibodies, Fc containing non-antibody therapeutic proteins of interest, antibody-drug conjugates (ADCs), and antibodies for reducing opsonization and/or immune cell-mediated clearance constitute preferred embodiments of the present invention. In advantageous embodiments, the EVs according to the present invention are coated with a plurality of proteins comprising an Fc domain. For instance, when using a highly expressed EV protein such as CD63 or CD81 or syntenin one can achieve very dense coating of the surface of EVs. Thus, the present invention may be coated with at least 10 proteins comprising an Fc domain, preferably at least 20 proteins comprising an Fc domain, even more preferably at least 30 proteins comprising an Fc domain. Such proteins may be copies of the same protein (e.g. 50 etanercept molecules coating one EV, by way of example) or more than one protein (e.g. 30 etanercept molecules and 30 gp130-Fc domain fusion proteins coating one EV, by way of example). By selecting an optimal combination of EV protein and Fc binder it may be possibly to increase the display further, in certain cases it may be possible to coat an EV with more than 50 proteins comprising an Fc domain, or even more than approximately 75 proteins comprising an Fc domain.

In a further aspect, the present invention also relates to cells comprising a fusion protein between a display polypeptide and an Fc binding polypeptide. The display polypeptide may typically be a transmembrane protein. Various exosomal polypeptides can be used for this purpose, and so can regular cellular

transmembrane proteins such as T cell receptor transmembrane domains, interleukin receptors, transferrin receptor, stannin, sarcolipin, phospholamban, and various other membrane proteins such as channels and symporters/importers, which can transport the Fc binding polypeptide to the cell membrane for display on the outside or on the inside of the cell membrane, depending on whether the Fc containing protein of interest is going to be present inside the cell or displayed on the surface of the cell. In a preferred embodiment, the Fc containing protein is an antibody and the cell is a cell intended for cellular therapy, such as a chimeric antigen receptor (CAR) T cell. Using this approach, a cell could be targeted to a particular tissue of interest, in the case of a CAR-T cell the tissue of interest is likely to be tumor tissue. In the case of an immune-modulatory cell such as an MSC, the tissue of interest is likely to be a site of inflammation and/or injury. All antibodies and other Fc containing proteins herein are equally suitable for being displayed on a cell for the same reasons as for the EVs.

In another aspect, the present invention relates to a complex formed between a fusion protein comprising (i) an exosomal polypeptide and (ii) an Fc binding polypeptide, and a protein comprising an Fc domain. EVs per the present invention thus typically comprise a plurality of such complexes, and typically the higher number of such complexes displayed on an EV without issues relating to for instance to steric hindrance the more potent the EV becomes from a therapeutic and/or targeting standpoint. Such fusion protein-Fc containing protein complexes are typically present in the lipid membrane of an EV, by virtue of the presence of the exosomal protein which mediates transport and anchoring into exosomal membranes. However, the complexes may also be present in other lipid membranes, for instance cell membranes or membranes of cell organelles. The EVs as per the present invention also form a type of nanoparticle complex with the Fc containing proteins. Typically, as above-mentioned, each EV comprising the Fc binding polypeptide binds to a plurality of Fc containing proteins, which results in EVs that are coated (decorated) and/or contains a plurality of the Fc containing protein of interest. An example of this is that Fc-binding EVs that are allowed to

bind to antibodies through incubation with such antibodies become decorated on their surface with a plurality of antibodies, e.g. at least 10 antibodies per EV, or more frequently at least 20 or 30 antibodies per EV. Thus, this EV-antibody complex constitutes a type of nanoparticle complex with significant utility for targeting, therapy, and intracellular delivery.

Importantly, the present invention also provides for a method of loading Fc containing proteins into EVs in an endogenous fashion. Such endogenous loading methods comprise co-expression in an EV-producing cell of (i) a fusion protein comprising an exosomal polypeptide and an Fc binding polypeptide, and (ii) a protein of interest comprising an Fc domain. The exosomal polypeptide fused to the Fc binding domain enables active sorting of the fusion protein into EVs in the EV-producing cell, and the presence of the Fc binding protein enables the fusion protein to drag along (transport) the Fc containing protein into an EV. As above-mentioned, various exosomal proteins can be used to sort fusion proteins into EVs and especially in the case of endogenous loading the choice of protein is highly important. Soluble EV proteins such as Alix and syntenin may be suitable for the loading of Fc containing proteins that are meant to be soluble in the target environment, such as Cas9 which preferably is soluble inside a target cell. However, endogenous loading of Fc containing proteins can also be achieved with transmembrane and/or membrane-associated EV proteins such as CD9, CD81, CD63, etc. When utilizing membrane-bound exosomal proteins for endogenous loading the Fc binding polypeptide is instead placed on a loop on the luminal side of the EV membrane as opposed to on the extravesicular side, enabling luminal as opposed to extravesicular loading. The EVs as per the present invention thus normally comprise a plurality of such fusion protein-Fc domain-containing protein complexes (“a plurality” shall be understood to relate to multiple copies of the same Fc domain-containing protein or several different Fc domain containing proteins), either anchored in or associated with the EV membrane on the inside or on the outside, or in essentially soluble form luminally in the EV.

In a further aspect, the present invention relates to methods for producing EVs capable of binding to proteins comprising an Fc domain, such as antibodies and proteins engineered to comprise Fc domains. Such methods typically comprise the steps of (i) introducing into an EV source cell a polynucleotide construct which encodes a fusion protein comprising at least one Fc binding polypeptide and at least one exosomal polypeptide, and (ii) collecting EVs that have been secreted by the EV-producing source cells, wherein the EVs comprise the fusion protein which has been expressed from the polynucleotide construct. In a subsequent step, the EVs comprising the fusion protein may be purified using a suitable purification technique, followed by being exposed to an Fc domain-containing protein, such as an antibody, to enable binding of the Fc containing protein through interaction between the Fc binding polypeptide and the Fc domain of the protein of interest. As above-mentioned, in an alternative embodiment, step (i) may also include expressing the Fc containing protein of interest from a polynucleotide construct in the same EV source cell, thereby achieving endogenous loading of the EV. The fusion protein (between the exosomal polypeptide and the Fc binding polypeptide) and the Fc containing protein may be expressed from the same or from different polynucleotide construct, depending on the construct design.

In yet another aspect, the present invention relates to a method for attaching at least one protein comprising an Fc domain, such as an antibody, to an EV. In an embodiment, the method is for coating an EV with said at least one protein comprising an Fc domain. Such methods comprise the steps of (i) providing an EV comprising a fusion protein comprising at least one Fc binding protein fused to at least one exosomal polypeptide, and (ii) allowing the Fc binding protein of the fusion protein to bind the Fc domain of at least one protein comprising an Fc domain. The EV source cells used for production of EVs comprising the fusion proteins as per the present invention may be either stably or transiently transfected with the polynucleotide construct needed to generate the EVs carrying the fusion protein-Fc containing protein complex. Stable transfection is advantageous as it enables creation of master cell banks (MCBs) and working cell banks (WCBs).

However, transient transfection is also advantageous in certain instances, for example when assessing different constructs or e.g. when rapidly creating an autologous therapy comprising EVs obtained from a patient's own EV-producing cells.

In a further aspect, the present invention relates to methods for delivery of a protein of interest into the intracellular environment of target cells, either in vitro, ex vivo or in vivo. Such methods comprise the steps of (i) providing EVs comprising at least one Fc binding polypeptide, (ii) putting the EV comprising the Fc binding polypeptide in contact with an Fc containing protein, in order to enable binding between the Fc binding polypeptide comprised in the EV and the Fc domain of the Fc containing protein, and (iii) putting the complex formed between the EV and the Fc containing protein(s) (i.e. an EV having at least one Fc containing protein attached to it through the interaction between the Fc binding polypeptide and the Fc domain) in contact with the target cell(s). In a preferred embodiment, the Fc binding polypeptide is comprised in a fusion protein together with at least an exosomal polypeptide, but optionally other polypeptides and domains as well. Due to the unique ability of EVs to internalize into target cells, the Fc containing protein will also be internalized. This is highly advantageous and opens up the intracellular space for delivery of virtually any protein-based therapeutics. As above-mentioned, the EV-mediated internalization of Fc containing proteins take place both in vivo and in vitro, and these methods may thus have significant impact for therapy development but also for research and diagnostic purposes.

In a further aspect, the present invention pertains to the use of EVs as delivery vehicles for antibodies and other Fc domain containing proteins of interest. Furthermore, the present invention also provides for the use of EVs attached to Fc containing proteins as delivery vehicles for other therapeutic agents of interest. As a non-limiting example, EVs having attached to their surface a targeting antibody may also contain additional therapeutic agents which may be present either inside the EV and/or in the EV membrane. For instance, an antibody-coated EV may be

used to deliver an RNA therapeutics cargo (such as an mRNA, an siRNA, an oligonucleotide, etc.) to a target cell, tissue and/or organ. In another non-limiting example, EVs having attached to their surface an Fc containing targeting protein (such as a scFv which have been engineered to contain an Fc domain) and comprising inside the EV or in/on the EV membrane a therapeutic protein such as Cas9 for gene editing, optionally together with a CRISPR RNA guide strand.

In further aspects, the methods of the present invention may also comprise exposing the EV source cells to serum starvation, hypoxia, bafilomycin, or cytokines such as TNF-alpha and/or IFN-gamma, in order to influence the yield or properties of the resulting EVs. The EV production scale and timeline will be heavily dependent on the EV-producing cell or cell line and may thus be adapted accordingly by a person skilled in the art. The methods as per the present invention may further comprise an EV purification step, which may be carried out prior to co-incubating the EVs comprising the fusion protein with the Fc domain-containing protein (such as an antibody) to be attached to (e.g. coated on) the EVs. EVs may be purified through a procedure selected from a group of techniques comprising liquid chromatography (LC), high-performance liquid chromatography (HPLC), bead-eluate chromatography, spin filtration, tangential flow filtration (TFF), hollow fiber filtration, centrifugation, immunoprecipitation, flow field fractionation, dialysis, microfluidic-based separation, etc., or any combination thereof. In an advantageous embodiment, the purification of the EVs is carried out using a sequential combination of filtration (preferably ultrafiltration (UF), tangential flow filtration or hollow fiber filtration) and size exclusion liquid chromatography (LC) or bead-eluate chromatography. This combination of purification steps results in optimized purification, which in turn leads to superior therapeutic activity. Further, as compared to ultracentrifugation (UC), which is routinely employed for purifying exosomes, sequential filtration-chromatography is considerably faster and possible to scale to higher manufacturing volumes, which is a significant drawback of the current UC methodology that dominates the prior art. Another advantageous purification methodology is tangential flow filtration (TFF), which offers scalability

and purity, and which may be combined with any other type of purification technique.

In yet another aspect, the present invention pertains to pharmaceutical compositions comprising EVs, normally in the form of populations of EVs, as per the present invention. Typically, the pharmaceutical compositions as per the present invention comprise one type of therapeutic EV (i.e. a population of EVs comprising a certain type of fusion protein and being coated by one or more types of Fc containing proteins, such as antibodies; Cas9-Fc fusion proteins and Fc-RNP fusion complexes; lysosomal storage disorder enzymes fused to Fc domains, etc.) formulated with at least one pharmaceutically acceptable excipient. However, more than one type of EV population may naturally be comprised in a single pharmaceutical composition, for instance in cases where a combinatorial antibody treatment is desirable. Naturally however, as above-mentioned, a single EV or a single population of EVs may comprise more than one Fc-containing protein (e.g. an antibody) bound to the EV surface. The at least one pharmaceutically acceptable excipient may be selected from the group comprising any pharmaceutically acceptable material, composition or vehicle, for instance a solid or liquid filler, a diluent, an excipient, a carrier, a solvent or an encapsulating material, which may be involved in e.g. suspending, maintaining the activity of or carrying or transporting the EV population from one organ, or portion of the body, to another organ, or portion of the body (e.g. from the blood to any tissue and/or organ and/or body part of interest).

The present invention also relates to cosmetic applications of EVs. Thus, the present invention may pertain to skin care products such as creams, lotions, gels, emulsions, ointments, pastes, powders, liniments, sunscreens, shampoos, etc., comprising a suitable EV, in order to improve and/or alleviate symptoms and problems such as dry skin, wrinkles, folds, ridges, and/or skin creases. In one embodiment, EVs (carrying a fusion protein bound to e.g. an antibody of interest) are obtained from a suitable EV-producing cell source with regenerative properties (for instance an MSC) are comprised in a cosmetic cream, lotion, or gel for use in

the cosmetic or therapeutic alleviation of wrinkles, lines, folds, ridges and/or skin creases.

In yet another aspect, the present invention relates to EVs as per the present invention for use in medicine. Naturally, when an EV comprising a fusion protein bound to an Fc domain of a protein of interest (such as an antibody) is used in medicine, it is in fact normally a population of EVs that is being used. The dose of EVs administered to a patient will depend on the number of e.g. antibodies of interest that has been coated on the EV surface, the disease or the symptoms to be treated or alleviated, the administration route, the pharmacological action of the therapeutic protein itself, the inherent properties of the EVs, the presence of any targeting antibodies or other targeting entities, as well as various other parameters of relevance known to a skilled person.

The EVs of the present invention carrying the Fc containing proteins may be used for several different therapeutic and pharmaceutical aspects. In one embodiment, the EVs are covered with antibodies or other Fc containing proteins that target a specific cell type, tissue, and/or organ. This is a highly powerful way of targeting EVs, which may comprise other pharmaceutical agents in addition to the Fc containing protein, to tissues of interest, and could represent a step chance in targeted drug delivery. In another embodiment, therapeutic antibodies or other Fc domain-containing proteins that interact with a target antigen of interest can be efficiently delivered to tissues of interest that are typically hard to reach, using EVs with ability to cross biological barriers. This approach may for instance enable delivery of monoclonal antibodies into the central nervous system or into the brain. In yet another embodiment, coating of EVs with Fc containing proteins is a way of multiplexing the Fc containing protein of interest, in order to enhance or influence its target avidity or the conformation of its binding to a target of interest. In yet another embodiment, the EVs as per the present invention enable improved delivery and efficacy of antibody-drug conjugates (ADCs) or receptor-drug conjugates, as multiplexing of ADCs may significantly enhance their therapeutic

efficacy and their presence on EVs means they can also enter target cells. In a further embodiment, the ability of EVs to enter target cells means that the EVs of the present invention opens up the entire intracellular space and make it druggable by essentially any protein comprising an Fc domain and/or any protein onto which an Fc domain can be fused (such as an enzyme for enzyme replacement therapy, nuclease such as Cas9, or a tumor suppressor such as any one of p53, pVHL, APC, CD95, ST5, YPEL3, ST7, and ST14).

The EVs and the EV populations thereof as per the present invention may thus be used for prophylactic and/or therapeutic purposes, e.g. for use in the prophylaxis and/or treatment and/or alleviation of various diseases and disorders. A non-limiting sample of diseases wherein the EVs as per the present invention may be applied comprises Crohn's disease, ulcerative colitis, ankylosing spondylitis, rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, sarcoidosis, idiopathic pulmonary fibrosis, psoriasis, tumor necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS), deficiency of the interleukin-1 receptor antagonist (DIRA), endometriosis, autoimmune hepatitis, scleroderma, myositis, stroke, acute spinal cord injury, vasculitis, Guillain-Barré syndrome, acute myocardial infarction, ARDS, sepsis, meningitis, encephalitis, liver failure, non-alcoholic steatohepatitis (NASH), non-alcoholic fatty liver disease (NAFLD), kidney failure, heart failure or any acute or chronic organ failure and the associated underlying etiology, graft-vs-host disease, Duchenne muscular dystrophy and other muscular dystrophies, lysosomal storage diseases such as Gaucher disease, Fabry's disease, MPS I, II (Hunter syndrome), and III, Niemann-Pick disease, Pompe disease, etc., neurodegenerative diseases including Alzheimer´s disease, Parkinson´s disease, Huntington's disease and other trinucleotide repeat-related diseases, dementia, ALS, cancer-induced cachexia, anorexia, diabetes mellitus type 2, and various cancers. Virtually all types of cancer are relevant disease targets for the present invention, for instance, Acute lymphoblastic leukemia (ALL), Acute myeloid leukemia, Adrenocortical carcinoma, AIDS-related cancers, AIDS-related lymphoma, Anal cancer, Appendix cancer, Astrocytoma,

cerebellar or cerebral, Basal-cell carcinoma, Bile duct cancer, Bladder cancer, Bone tumor, Brainstem glioma, Brain cancer, Brain tumor (cerebellar astrocytoma, cerebral astrocytoma/malignant glioma, ependymoma, medulloblastoma, supratentorial primitive neuroectodermal tumors, visual pathway and hypothalamic glioma), Breast cancer, Bronchial adenomas/carcinoids, Burkitt's lymphoma, Carcinoid tumor (childhood, gastrointestinal), Carcinoma of unknown primary, Central nervous system lymphoma, Cerebellar astrocytoma/Malignant glioma, Cervical cancer, Chronic lymphocytic leukemia, Chronic myelogenous leukemia, Chronic myeloproliferative disorders, Colon Cancer, Cutaneous T-cell lymphoma, Desmoplastic small round cell tumor, Endometrial cancer, Ependymoma, Esophageal cancer, Extracranial germ cell tumor, Extragonadal Germ cell tumor, Extrahepatic bile duct cancer, Eye Cancer (Intraocular melanoma, Retinoblastoma), Gallbladder cancer, Gastric (Stomach) cancer, Gastrointestinal Carcinoid Tumor, Gastrointestinal stromal tumor (GIST), Germ cell tumor (extracranial, extragonadal, or ovarian), Gestational trophoblastic tumor, Glioma (glioma of the brain stem, Cerebral Astrocytoma, Visual Pathway and Hypothalamic glioma), Gastric carcinoid, Hairy cell leukemia, Head and neck cancer, Heart cancer, Hepatocellular (liver) cancer, Hodgkin lymphoma, Hypopharyngeal cancer, Intraocular Melanoma, Islet Cell Carcinoma (Endocrine Pancreas), Kaposi sarcoma, Kidney cancer (renal cell cancer), Laryngeal Cancer, Leukemias ((acute lymphoblastic (also called acute lymphocytic leukemia), acute myeloid (also called acute myelogenous leukemia), chronic lymphocytic (also called chronic lymphocytic leukemia), chronic myelogenous (also called chronic myeloid leukemia), hairy cell leukemia)), Lip and Oral, Cavity Cancer, Liposarcoma, Liver Cancer (Primary), Lung Cancer (Non-Small Cell, Small Cell), Lymphomas, AIDS-related lymphoma, Burkitt lymphoma, cutaneous T-Cell lymphoma, Hodgkin lymphoma, Non-Hodgkin, Medulloblastoma, Merkel Cell Carcinoma, Mesothelioma, Metastatic Squamous Neck Cancer with Occult Primary, Mouth Cancer, Multiple Endocrine Neoplasia Syndrome, Multiple Myeloma/Plasma Cell Neoplasm, Mycosis Fungoides, Myelodysplastic/Myeloproliferative Diseases, Myelogenous Leukemia, Chronic

Myeloid Leukemia (Acute, Chronic), Myeloma, Nasal cavity and paranasal sinus cancer, Nasopharyngeal carcinoma, Neuroblastoma, Oral Cancer, Oropharyngeal cancer, Osteosarcoma/malignant fibrous histiocytoma of bone, Ovarian cancer, Ovarian epithelial cancer (Surface epithelial-stromal tumor), Ovarian germ cell tumor, Ovarian low malignant potential tumor, Pancreatic cancer, Pancreatic islet cell cancer, Parathyroid cancer, Penile cancer, Pharyngeal cancer, Pheochromocytoma, Pineal astrocytoma, Pineal germinoma, Pineoblastoma and supratentorial primitive neuroectodermal tumors, Pituitary adenoma, Pleuropulmonary blastoma, Prostate cancer, Rectal cancer, Renal cell carcinoma (kidney cancer), Retinoblastoma, Rhabdomyosarcoma, Salivary gland cancer, Sarcoma (Ewing family of tumors sarcoma, Kaposi sarcoma, soft tissue sarcoma, uterine sarcoma), Sézary syndrome, Skin cancer (nonmelanoma, melanoma), Small intestine cancer, Squamous cell, Squamous neck cancer, Stomach cancer, Supratentorial primitive neuroectodermal tumor, Testicular cancer, Throat cancer, Thymoma and Thymic carcinoma, Thyroid cancer, Transitional cell cancer of the renal pelvis and ureter, Urethral cancer, Uterine cancer, Uterine sarcoma, Vaginal cancer, Vulvar cancer, Waldenström macroglobulinemia, and/or Wilm's tumor.

The EVs as per the present invention may be administered to a human or animal subject via various different administration routes, for instance auricular (otic), buccal, conjunctival, cutaneous, dental, electro-osmosis, endocervical, endosinusial, endotracheal, enteral, epidural, extra-amniotic, extracorporeal, hemodialysis, infiltration, interstitial, intra-abdominal, intra-amniotic, intra-arterial, intra-articular, intrabiliary, intrabronchial, intrabursal, intracardiac, intracartilaginous, intracaudal, intracavernous, intracavitary, intracerebral, intracisternal, intracorneal, intracoronal (dental), intracoronary, intracorporus cavernosum, intradermal, intradiscal, intraductal, intraduodenal, intradural, intraepidermal, intraesophageal, intragastric, intragingival, intraileal, intralesional, intraluminal, intralymphatic, intramedullary, intrameningeal, intramuscular, intraocular, intraovarian, intrapericardial, intraperitoneal, intrapleural, intraprostatic, intrapulmonary, intrasinal, intraspinal, intrasynovial, intratendinous,

intratesticular, intrathecal, intrathoracic, intratubular, intratumor, intratym panic, intrauterine, intravascular, intravenous, intravenous bolus, intravenous drip, intraventricular, intravesical, intravitreal, iontophoresis, irrigation, laryngeal, nasal, nasogastric, occlusive dressing technique, ophthalmic, oral, oropharyngeal, other, parenteral, percutaneous, periarticular, peridural, perineural, periodontal, rectal, respiratory (inhalation), retrobulbar, soft tissue, subarachnoid, subconjunctival, subcutaneous, sublingual, submucosal, topical, transdermal, transmucosal, transplacental, transtracheal, transtympanic, ureteral, urethral, and/or vaginal administration, and/or any combination of the above administration routes, which typically depends on the disease to be treated and/or the characteristics of the antibody or the EV population as such.

Suitable targeting antibodies in accordance with the present invention may target antigens deriving from e.g. tumors, solid organs, bodily structures, cell or tissue types. Non-limiting examples of the origin of antigens that may be targeted by targeting antibodies include liver, lung, kidney, heart, pancreas, adrenal glands, thyroid glands, parathyroid glands, brain including all brain regions (for instance thalamus, hypothalamus, striatum, etc.), the blood-brain-barrier, the CNS, the PNS, bone marrow, the skin, the vascular system, the lymphatic system including the spleen, joints, eyes, muscle tissues, sites of inflammation, sites of injury, and cell types such as adipocytes, muscle cells (myoblasts and myotubes), satellite cells, cardiac cells, endothelial cells, fibroblasts, hepatocytes, renal cells, pericytes, neurons, glia cells, astrocytes, oligodendrocytes, macrophages, DC-cells, B-cells, T-cells, NK-cells, chrondrocytes, osteoblast, osteocytes, epithelial cells, erythrocytes, earlier progenitors such as multipotential hematopoietic stem cells/hemocytoblasts, myeloid progenitors, lymphoid progenitors, etc. Non-limiting examples of antigens relevant for targeting cancer includes adenocarcinoma antigen, alpha-fetoprotein, BAFF, C242 antigen, CA-125, carbonic anhydrase 9 (CA-IX), disialoganglioside (GD2), 4-IBB, 5T4, CD22, CD221, CD23 (IgE receptor), CD28, CD30 (TNFRSF8), CD33, CD4, CD40, CD44 v6, CD51, CD52, CD56, CD74, CD80, CEA, CNT0888, CTLA-4, DR5, EGFR, EpCAM, CD3, FAP,

fibronectin extra domain-B, folate receptor 1, GD2, GD3 ganglioside, glycoprotein 75, GPNMB, HER2/neu, HGF, human scatter factor receptor kinase, IGF-1 receptor, IGF-I, IgGl, LI-CAM, IL-13, IL-6, insulin-like growth factor I receptor, integrin α5β1, integrin αvβ3, legumain, MORAb-009, MS4A1, MUC1, mucin CanAg, C-MET, CCR4, CD 152, CD 10, CD 19, CD20, CD200, N-glycolylneuraminic acid, NPC-IC, PDGF-R a, PDL192, phosphatidylserine, tumor antigen CTAA16.88, VEGF-A, VEGFR-1 , VEGFR2, vimentin, RANKL, RON, ROR1, SCH 900105, SDC1, SLAMF7, TAG-72, tenascin C, TGF beta 2, TGF-β, TRAIL-R1, TRAIL-R2, folic acid receptor, transferrin receptors and any combination thereof.

Antibodies or other Fc domain-containing proteins coating EVs in order to prevent or reduce opsonization and/or immune cell-mediated EV clearance in accordance with the present invention may be essentially any Fc-containing protein, such as an antibody, that is present naturally in serum or in any other body fluid. Such antibodies may thus be of any type, e.g. IgG, IgA, IgM, IgE and/or IgD, but any other protein comprising an Fc domain and having the capacity to prevent opsonization of EVs *in vivo* may also be used to coat the EV. Thus, coating of EVs to avoid or reduce clearance by immune may be carried out actively or passively, wherein the active coating may involve decorating the EV in question with a suitable protein comprising an Fc domain prior to application *in vivo*. Suitable Fc containing proteins for this purpose include Factor H and/or Factor I fused to an Fc domain. Factor H and Factor I are negative regulators of the complement system (by inhibiting C3 conversion to C3b), thereby resulting in reduced EV clearance when fused to an Fc domain and attached to the EV surface through the binding between the Fc binding polypeptide and the Fc domain of the fusion proteins. Passive coating on the other hand may entail allowing the EV in question to sequester proteins *in vivo* with its available non-bound Fc binders.

In a further embodiment, suitable non-limiting examples of therapeutic and/or targeting antibodies in accordance with the present invention may be any one or more of Abagovomab, Abciximab, Actoxumab, Adalimumab, Adecatumumab,

Adotrastuzumab emtansine, Aducanumab, Afelimomab, Afutuzumab, Alacizumab Alemtuzumab, Alirocumab, Altumomab pentetate, Amatuximab, Anatumomab mafenatox, Anifrolumab, Anrukinzumab, Apolizumab, Arcitumomab, Aselizumab, Atezolizumab, Atinumab, Atlizumab, Atorolimuma, Avelumab, Bapineuzumab, Basiliximab, Bavituximab, Bectumomab, Belimumab, Benralizumab, Bertilimumab, Besilesomab, Bevacizumab, Bezlotoxumab, Biciromab, Bimagrumab, Bivatuzumab mertansine, Blinatumomab, Blosozumab, Brentuximab vedotin, Briakinumab, Brodalumab, Canakinumab, Cantuzumab mertansine, Cantuzumab ravtansine, Caplacizumab, Capromab pendetide, Carlumab, Catumaxomab, cBR96-doxorubicin immunoconjugate, Cedelizumab, Certolizumab pegol, Cetuximab, Citatuzumab bogatox, Cixutumumab, Clazakizumab, Clenoliximab, Clivatuzumab tetraxetan, Conatumumab, Concizumab, Crenezumab, CR6261, Dacetuzumab, Daclizumab, Dalotuzumab, Daratumumab, Demcizumab, Denosumab, Detumomab, Dinutuximab, Dorlimomab aritox, Drozitumab, Duligotumab, Dupilumab, Durvalumab, Dusigitumab, Ecromeximab, Eculizumab, Edobacomab, Edrecolomab, Efalizumab, Efungumab, Eldelumab, Elotuzumab, Elsilimomab, Enavatuzumab, Enlimomab pegol, Enokizumab, Enoticumab, Ensituximab, Epitumomab cituxetan, Epratuzumab, Erlizumab, Ertumaxomab, Etanercept, Etaracizumab, Etrolizumab, Evolocumab, Exbivirumab, Fanolesomab, Faralimomab, Farletuzumab, Fasinumab, FBTA05, Felvizumab, Fezakinumab, Ficlatuzumab, Figitumumab, Flanvotumab, Fontolizumab, Foralumab, Foravirumab, Fresolimumab, Fulranumab, Futuximab, Galiximab, Ganitumab, Gantenerumab, Gavilimomab, Gemtuzumab ozogamicin, Gevokizumab, Girentuximab, Glembatumumab vedotin, Golimumab, Gomiliximab, Guselkumab, Ibalizumab, Ibritumomab tiuxetan, Icrucumab, Igovomab, IMAB362, Imciromab, Imgatuzumab, Inclacumab, Indatuximab ravtansine, Infliximab, Intetumumab, Inolimomab, Inotuzumab ozogamicin, Ipilimumab, Iratumumab, Itolizumab, Ixekizumab, Keliximab, Labetuzumab, Lambrolizumab, Lampalizumab, Lebrikizumab, Lemalesomab, Lerdelimumab, Lexatumumab, Libivirumab, Ligelizumab, Lintuzumab, Lirilumab, Lodelcizumab, Lorvotuzumab mertansine, Lucatumumab, Lumiliximab,

Mapatumumab, Margetuximab, Maslimomab, Mavrilimumab, Matuzumab, Mepolizumab, Metelimumab, Milatuzumab, Minretumomab, Mitumomab, Mogamulizumab, Morolimumab Motavizumab, Moxetumomab pasudotox, Muromonab-CD3, Nacolomab tafenatox, Namilumab, Naptumomab estafenatox, Narnatumab, Natalizumab, Nebacumab, Necitumumab, Nerelimomab, Nesvacumab, Nimotuzumab, Nivolumab, Nofetumomab merpentan, Ocaratuzumab, Ocrelizumab, Odulimomab, Ofatumumab, Olaratumab, Olokizumab, Omalizumab, Onartuzumab, Ontuxizumab, Oportuzumab monatox, Oregovomab, Orticumab, Otelixizumab, Otlertuzumab, Oxelumab, Ozanezumab, Ozoralizumab, Pagibaximab, Palivizumab, Panitumumab, Pankomab, Panobacumab, Parsatuzumab, Pascolizumab, Pateclizumab, Patritumab, Pembrolizumab, Pemtumomab, Perakizumab, Pertuzumab, Pexelizumab, Pidilizumab, Pinatuzumab vedotin, Pintumomab, Placulumab, Polatuzumab vedotin, Ponezumab, Priliximab, Pritoxaximab, Pritumumab, PRO 140, Quilizumab, Racotumomab, Radretumab, Rafivirumab, Ramucirumab, Ranibizumab, Raxibacumab, Regavirumab, Reslizumab, Rilotumumab, Risankizumab, Rituximab, Robatumumab, Roledumab, Romosozumab, Rontalizumab, Rovelizumab, Ruplizumab, Samalizumab, Sarilumab, Satumomab pendetide, Secukinumab, Seribantumab, Setoxaximab, Sevirumab, Sibrotuzumab, Sifalimumab, Siltuximab, Simtuzumab, Siplizumab, Sirukumab, Solanezumab, Solitomab, Sonepcizumab, Sontuzumab, Stamulumab, Sulesomab, Suvizumab, Tabalumab, Tacatuzumab tetraxetan, Tadocizumab, Talizumab , Tanezumab, Taplitumomab paptox, Tefibazumab, Telimomab aritox, Tenatumomab, Teneliximab, Teplizumab, Teprotumumab, Ticilimumab, Tildrakizumab, Tigatuzumab, Tocilizumab, Toralizumab, Tositumomab, Bexxar, Tovetumab, Tralokinumab, Trastuzumab, Tregalizumab, Tremelimumab, Tucotuzumab celmoleukin, Tuvirumab, Ublituximab, Urelumab, Urtoxazumab, Ustekinumab, Vantictumab, Vapaliximab, Vatelizumab, Vedolizumab,Veltuzumab, Vepalimomab, Vesencumab, Visilizumab, Volociximab, Vorsetuzumab mafodotin, Votumumab, Zalutumumab, Zanolimumab, Zatuximab, Ziralimumab, Zolimomab aritox, or any combination thereof. Importantly, the present invention provides for

the delivery of any antibody, regardless of whether its target is intracellular and/or extracellular. The ability of the EVs of the present invention to efficiently internalize antibodies represent a step-change in monoclonal antibody development and may enable targeting antigens and targets across the entire intracellular compartment of target cells. Importantly, antibodies and Fc containing proteins as per the present invention may advantageously be labelled for detection and imaging purposes, for instance using fluorophores, MRI agents, PET agents, radioactive agents, enzymes, nanoparticles, metals, organic and inorganic compounds, etc.

It shall be understood that the above described exemplifying aspects, embodiments, alternatives, and variants can be modified without departing from the scope of the invention. The invention will now be further exemplified with the enclosed examples, which naturally also can be modified considerably without departing from the scope and the gist of the invention.

Experimental part

Materials and methods

Construct design and cloning: Various fusion proteins comprising at least one exosomal polypeptide and at least one Fc binding polypeptide have been constructed, cloned into vectors and produced in several different EV-producing cell sources. ORFs were typically generated by synthesis and cloned into the mammalian expression vector pSF-CAG-Amp. Briefly, synthesized DNA and vector plasmid were digested with enzymes NotI and SalI as per manufacturers instruction (NEB). Restricted, purified DNA fragments were ligated together using T4 ligase as per manufacturers instruction (NEB). Successful ligation events were selected for by bacterial transformation on ampicillin-supplemented plates. Plasmid for transfection was generated by 'maxi-prep', as per manufacturers instruction.

In cases where Fc containing proteins were endogenously produced in the same EV-producing cell source that expresses the fusion protein, ORF sequences were purchased (Origene Technologies, Inc.) and amplified and cloned into the MSC A site of pIRES bicistronic expression vector (Clonetech, Laboratories Inc.) such that upon translation the exosomal polypeptide was fused to the Fc binding polypeptide in one construct, whereas the Fc containing protein of interest was translated separately (from a separate construct or from the same construct) and transported into the EV to be formed in the EV-producing cell source. Most of the cloning was performed using the NEBuilder HiFi DNA Assembly Cloning Kit (NEB, Inc.) and confirmed using Sanger sequencing (Source BioScience). The pIRES vector enables bicistronic expression of both transgenes simultaneously, ensuring EV-producing cells would express both the fusion protein and the Fc containing protein of interest simultaneously. Plasmids were transformed into the NEB 5-alpha Competent E.coli cells (NEB, Inc.) and grown overnight in a shaking incubator according to manufacturer's recommendations. Plasmids were isolated and purified using the 'maxi-prep plasmid DNA purification kit' (Qiagen), as per manufacturer's instruction.

- Cell culture and transfection

Depending on the experimental design and assays, in certain cases, non-viral transient transfection and exosome production was carried out in conventional 2D cell culture, whereas in other cases virus-mediated transduction was employed to create stable cell lines, which were typically cultured in bioreactors of different type. For conciseness, only a few examples are mentioned herein.

HEK293T cells were typically seeded into 15 cm dishes ($9x10^6$ cells per dish) and left overnight in serum-containing DMEM as recommended by ATCC. The following day the cells were transiently transfected with lipoplexed DNA added directly onto cells. Briefly, DNA and polyethyleneimine (PEI) were separately

incubated in OptiMEM for 5 minutes before combining together for 20 minutes at room temperature. Lipoplexed DNA and cells were co-incubated for 6 hours following which conditioned culture media was changed to OptiMEM for 48 hours. Other cells and cell lines that were evaluated in dishes, flasks and other cell culture vessels included bone marrow-derived mesenchymal stromal cells (BM-MSCs) and Wharton's jelly-derived MSCs (WJ-MSCs), amnion cells, fibroblasts, various endothelial and epithelial cells, as well as various immune cells and cell lines.

In the case of viral transduction and creation of stable cell lines for various combinations of fusion proteins and Fc containing proteins of interest, cell sources such as BM-MSCs, WJ-MSC, fibroblasts, amnion cells, fibroblasts, various endothelial and epithelial cells, were virus-transduced, typically using lentivirus (LV). Typically, 24 hours before infection, 100.000 cells (e.g. fibroblasts, MSCs, etc.) or 200.000 cells (e.g. HEK293T) are plated in a 6-well plate. 2 uL of LV and optionally Polybrene (or hexadimethrine bromide, final concentration on the well of 8 ug/mL) are added, and 24 hours post transduction the cell medium of transduced cells is changed to fresh complete media. At 72 hours post transduction, puromycin selection (4-6µg/ml) is performed, normally for 7 days followed by analysis of stable expression of the fusion protein construct comprising the exosomal polypeptide and the Fc binding polypeptide.

Stable cells were cultured in either 2D culture or in bioreactors, typically hollow-fiber bioreactors or stir-rank bioreactors, and conditioned media was subsequently harvested for exosome preparation. Various preparation and purification steps were carried out. The standard workflow comprises the steps of pre-clearing of the supernatant, filtration-based concentration, chromatography-based removal of protein contaminants, and optional formulation of the resultant exosome composition in a suitable buffer for *in vitro* and/or *in vivo* assays.

- Assays and analytics

Western blot is a highly convenient analytical method to evaluate the enrichment of fusion proteins in EVs. Briefly, SDS-PAGE was performed according to manufacturer's instruction (Invitrogen, Novex PAGE 4-12% gels), whereby 1 x 10^{10} exosomes and 20 ug cell lysate were loaded per well. Proteins from the SDS-PAGE gel were transferred to PVDF membrane according to manufacturer's instruction (Immobilon, Invitrogen). Membranes were blocked in Odyssey blocking buffer (Licor) and probed with antibodies against the Fc binding polypeptide and/or the exosomal protein according to supplier's instruction (Primary antibodies – Abcam, Secondary antibodies – Licor). Molecular probes visualized at 680 and 800 nm wavelengths.

For EV size determination, nanoparticle tracking analysis (NTA) was performed with a NanoSight instrument equipped with analytical software. For all recordings, we used a camera level of 13 or 15 and automatic function for all post-acquisition settings. Electron microscopy and fluorescence microscopy were frequently used to understand intracellular location and release and to quantitate and analyze EVs.

EVs were isolated and purified using a variety of methods, typically a combination of filtration such as TFF and LC, in particular bead-elute LC. Typically, EV-containing media was collected and subjected to a low speed spin at 300*g* for 5 minutes, followed by 2000*g* spin for 10 minutes to remove larger particles and cell debris. The supernatant was then filtered with a 0.22 µm syringe filter and subjected to different purification steps. Large volumes were diafiltrated and concentrated to roughly 20 ml using the Vivaflow 50R tangential flow (TFF) device (Sartorius) with 100 kDa cutoff filters or the KR2i TFF system (SpectrumLabs) with 100 or 300 kDa cutoff hollow fibre filters. The preconcentrated medium was subsequently loaded onto the bead-eluate columns (HiScreen or HiTrap Capto Core 700 column, GE Healthcare Life Sciences), connected to an ÄKTAprime plus or ÄKTA Pure 25 chromatography system (GE Healthcare Life Sciences). Flow

rate settings for column equilibration, sample loading and column cleaning in place procedure were chosen according to the manufacturer's instructions. The sample was collected according to the UV absorbance chromatogram and concentrated using an Amicon Ultra-15 10 kDa molecular weight cut-off spin-filter (Millipore) to a final volume of 100 µl and stored at -80°C for further downstream analysis. To assess the protein and RNA elution profiles, media was concentrated and diafiltrated with KR2i TFF system using 100 kDa and 300 kDa hollow fibre filters and a sample analysed on a Tricorn 10/300 Sepharose 4 Fast Flow (S4FF) column (GE Healthcare Life Sciences).

- Examples

Example 1: Binding of IgG to EVs comprising Fc binding polypeptides (Fc-binding EVs)

EVs were isolated from the conditioned medium from engineered HEK293T cells (control versus Fc-binding construct that stably express Gp130 Extracellular domain-2XGGGGS linker-Z domain-Gp130 transmembrane domain-Leucine Zipper-N terminal syntenin-His tag) using tangential flow filtration with 300 kd hollow fiber columns, followed by ultrafiltration using 10 kd spin filters for concentration. The binding capacity for IgG by the Fc-binding EVs were then assessed using electron microscopy and flow cytometry.

For electron microscopy, 1 x 10^9 EVs were incubated with Rabbit anti-goat 10nm antibody conjugated with gold Nanoparticles for 2 h at 37°C. As shown in Figure 2, Fc-binding EVs (A) are decorated with nanogold labeled antibodies (i.e. Fc containing proteins), whereas control EVs (B) do not have any antibodies bound.

For flow cytometry, 1 x 10^8 EVs were incubated overnight on an orbital shaker at 450 rpm for 16 hours in 120 µl PBS with 15 µl antibody-coated capture beads from the MACSPlex Exosome Kit, human (Miltenyi Biotec, Order no 130-108-813). After washing, 3 µg of AlexaFluor647-conjugated human IgG Fc fragments (Jackson

Laboratories, Catalogue 009-600-008) were added to controls without EVs (A), control EVs (B), or Fc-binding EVs (Synthenin –gp130-zDomain-gp130) (C). After 1 hour incubation at room temperature, unbound Fc fragments were washed away and samples were analyzed via flow cytometry. In Figure 3 respective left dotplots show hard-dyed capture bead populations using B1-A (Excitation: 488 nm, Emission Filter: 500-550 nm; Area) versus B2-A (Excitation: 488 nm, Emission Filter: 565-605 nm, Area) parameters. Respective right plots show R1-A (Excitation: 635 nm, Emission Filter: 655-730 nm, Area) versus B2-A parameters, demonstrating binding of AlexaFluor647 labeled Fc-Fragments to EVs which have bound to the capture beads only in (C). Figure 3 shows that the Fc-binding EVs bind both to AlexaFluor647-labelled Fc fragments (human IgG) and very efficiently also to the Fc domains of all 39 different antibodies which are coated on all capture bead populations by the manufacturer included in the kit, including the two negative control bead populations.

Example 2: FCGR1A Lamp2B EVs for delivery of anti-HER2 antibody

EVs were isolated from HEK293T cells (either stably expressing FCGR1A Extracellular domain-4XGSlinker-Lamp2b or their wild type controls) using ultrafiltration and size exclusion chromatography. EVs were labelled with PKH26 red fluorescent dye, and decorated with anti-HER2 antibody or its isotype control by co-incubating EVs and antibody for 1 h at 37°C. Unbound antibody was removed by size exclusion chromatography. Uptake of antibody decorated EVs was characterized in HER2 low-expressing cell line MDA-MB-231 and in HER2 high-expressing cell line MDA-MB-361 using flow cytometry. Figure 4 shows that anti-HER2 antibody increases uptake of decorated EVs as compared to isotype control decorated and wild type EVs only in HER2 high-expressing cell line MDA-MB-361, but not in HER2 low-expressing cell line MDA-MB-231. Similar results were obtained with EVs expressing CD63-ZZ fusion proteins.

Example 3: Etanercept delivery by EVs comprising CD81-Protein A/G fusion proteins

EVs were isolated from HEK293T cells (either stably expressing CD81-ProteinA/G CD81 Second loop fusion protein or their wild type control) using ultrafiltration and size exclusion chromatography. EVs were decorated with etanercept or a control antibody by co-incubating EVs and etanercept for 1 h at 37°C. Unbound etanercept was removed by size exclusion chromatography. To study anti-inflammatory effect of the etanercept-decorated EVs, the well-studied TNBS-induced colitis mouse model was used. This model simulates the gut inflammation, cytokine storm and weight decrease associated with IBD patients. 24 mice were divided into four treatment groups, with 6 mice per group. The mice were pre-sensitized by applying 150 µl of a olive oil-acetate solution with 2% TNBS, on the skin, 1 week prior to colitis induction. Colitis was then induced by giving a rectal infusion of 100 µl solution containing 1.5 % TNBS in 40 % ethanol. Immediately post colitis induction, 10E10 EVs/g EVs in 200 µl were administrated intravenously in the tail vein. Figure 5 shows that etanercept-coated EVs protected mice from loss of body weight, as opposed to WT or control decorated-EVs, and displayed higher activity than naked etanercept, possible due to higher affinity between etanercept and TNFalpha when etanercept is multiplexed and/or when the Fc binding polypeptide binds to its Fc domain.

Example 4: Intracellular uptake and delivery of antibodies via Fc-binding EVs.

EVs were isolated from the conditioned medium of Wharton's jelly-derived MSCs (either stably expressing TNFR Extracellular domain-2XGGGGS linker-Z domain-TNFR transmembrane domain-foldon-N terminal syntenin fusion proteins or control) using ultrafiltration and size exclusion chromatography. In order to investigate whether Fc-binding EVs can be employed for intracellular delivery of Abs, 4 x 10^11 EVs were incubated in 400 µl for 16 hours (overnight) with total 3 µg AlexaFluor488-labelled anti-Lamin B2 IgGs [abcam ab200426, Rabbit

monocolonal EPR9701(B)]. For the uptake experiment, Huh7 cells were plated in 48 well plates at 30,000 cells per well and incubated for 16 hours before 0.675x10^11 antibody-labelled EVs were added. Cells were incubated for 2 hours at 37 °C and 5% CO_2 in a humidified atmosphere before they were trypsinized and analyzed by fluorescence microscopy (A) and flow cytometry (B) as shown in Figure 6; A) show green fluorescence signals merged with phase contrast images. Histograms in B) show the green fluorescence intensity on a logarithmic scale on the x-axis and the normalized frequency on the y-axis. 1: HuH7 cells not treated with any antibody or EVs. 2: HuH7 cells treated with control EVs which were incubated with anti-Lamin B2 antibodies. 3: HuH7 cells treated with Fc-binding EVs which were incubated with anti-Lamin B2 antibodies. Figure 6 shows that signals of fluorescent antibodies are clearly present in cells treated with Fc-binding EVs plus antibody, while fluorescence signals are absent in untreated (1) or control EV treated (2). This demonstrates that antibodies can be delivered intracellularly via Fc-binding EVs, and that binding to EVs dramatically increases uptake of antibodies into cells.

To demonstrate functional intracellular delivery, anti-NFkB antibodies (anti-NFkB-Ab) were incubated with respective EVs for 1 h at 37°C. A reporter cell line, HEK cells stably expressing NFkB-luciferase, were treated with 5 ng/ml hTNF-alpha and the EV-Ab-mix. After 6 hours of treatment the luciferase activity was measured. Figure 7 shows successful inhibition when the anti-NFkB-ab is delivered by Fc-binding EVs.

Example 5: EAE treatment using Fc-binding EVs decorated with anti-integrin-4-alpha-Ab

C57BL/6 mice were induced with experimental autoimmune encephalitis (EAE) by s.c. injection of 100 ul of MOG_{35-55}-CFA emulsion and i.p. injection of 400 ng pertussis toxin on the same day and two days following immunization. EVs were isolated as described in example 1, from conditioned media of bone marrow-

derived MSCs (either stably expressing CD9-ZZ (a fusion construct comprising as a non-limiting example the SEQ ID NO 1 operably linked to as a non-limiting example the SEQ ID NO 74) domain fusion proteins or their wild type controls). Anti-integrin-4-alpha antibodies (Ab) were incubated with respective EVs (zzEVs or ctrl-EVs). The EAE mice were injected with anti-integrin-4a-Ab with or without EVs at day 7. Figure 8 shows that ctrl-EVs, as well as anti-integrin-4a-Ab display moderate protective effect from EAE development per se, whereas zzEVs incubated with anti-integrin-4a-Ab displayed almost complete inhibition of EAE development.

Example 6: Targeted genomic deletion by delivering Fc-Cas9 endonuclease using Fc-binding EVs.

EVs were purified as stated in example 1 from cell culture medium obtained from adipocytes (stably transfected FCGR1A Extracellular domain-4XGSlinker-Lamp2b fusion protein or Lamp2b GFP as a control). Varying amounts of EVs was used (100 µg, 500 µg, 2.5 mg, 5 mg and 10 mg), whilst the amount of Fc (IGHG1) tagged-Cas9 Guide RNA complexes (so called RNP complexes) targeting HPRT remained at 100 µg. The final weight ratios of Cas9 complex to EVs were 1:1, 1:5, 1:25, 1:50 and 1:100 respectively and incubated at 22°C for 60 minutes. Maximum loading of Cas9 on EVs was obtained with 1:5 weight ratio of Cas9 complexes to EVs, free Cas9 complexes were removed by ultrafiltration. Then Cas9 loaded Fc+ Adipocytes-EVs were used to treat Huh7 cells with different concentration. After 24 hours cells were harvested and GeneArt Genomic Cleavage detection kit (Thermo scientific) was used as per manufacture protocol for preparing samples genomic cleavage assay and run on a 2% agarose gel. Indels were then quantified using Image J software. Figure 9 shows dose-dependent gene editing in target cells. Various other pairs of Fc binding polypeptides and Fc domains fused to Cas9 were also evaluated in the same setup. Human FCAMR (IgA and IgM binding), human FCGR3A (IgG binding), and human FCGRB (IgG binding) fused to CD83 and combined with Cas9 fused to several different human Fc domains (e.g. IGHM,

IGHA1, IGHG1, etc.) were also evaluated in the same setup. Overall, all construct showed gene editing activity but with lower potency than the FCGR1A Extracellular domain-4XGSlinker-Lamp2b fusion protein (data not shown).

Example 7: EV decoration with soluble lysosomal proteins using the human-Fc / pH-sensing Protein G complex

The C2 domain of Protein G fused to various exosomal proteins such as CD63, Lamp2, and the transferrin receptor were evaluated for their expression and EV display levels. To facilitate the tethering of a lysosomal protein to the surface of EVs, a human Fc domain (deriving from IgG) (hFc) was fused to either the N or C terminus of the enzyme GBA. In the case of the N-fusion construct, the hFc domain was inserted following the signal peptide native to GBA. Co-expression of both constructs led to a significant enrichment of GBA with EVs, more so than over expression of wild-type GBA. In addition to the wt C2 domain of Protein G, a pH-sensing C2 domain was also displayed on the EV surface in a manner previously described. While the pH sensing C2 domain has the capacity to effectively bind the human Fc region, the affinity for complex formation drops over a thousand-fold at pH 4. Following cellular uptake of GBA-decorated by pH-sensing C2 domain EVs, they are trafficked to the lysosomal compartment. Within the low pH of the compartment, the tethered GBA and pH-sensing c2 domain dissociate, facilitating the presence of free untethered GBA within the lumen of the lysosome.

Conditioned media isolated from Hek293T cells producing wild type exosomes, C2 domain of Protein G decorated exosomes or pH-sensing C2 domain of Protein G decorated exosomes was combined with conditioned media from Hek293T cells expressing wild type GBA or human Fc-GBA fusion for 1 hour at 37°C. In addition, a co-incubation was performed in which the conditioned media was acidified to pH 4. EVs were then isolated by ultrafiltration and bead elution chromatography. To assess the levels of exosome-tethered GBA, purified vesicles were analyzed by western blot and probed for the presence of GBA. To assess the GBA activity of

the GBA-carrying exosomes, patient-derived GBA deficient fibroblasts were co-incubated with GBA-enriched exosomes and analyzed for glucosylceramide levels 48 hours later. Figure 10 shows the outcome of this experiment: WT – wildtype Hek293t exosomes; GBA – wildtype GBA; hFc-GBA – human Fc fragment-GBA fusion; PG – exosomes decorated with the C2 domain of Protein G; PGpH – exosomes decorated with the C2 domain of pH sensing Protein G. n=3. In an analogous experiment, Protein L and Protein LG were also evaluated as Fc binding polypeptides in the same setup, resulting in similar outcomes (data not shown).

Example 8: EVs coated with siRNA-loaded Ago2 fused to a human Fc domain

EVs comprising fusion proteins between Lamp2b and the Fc binding polypeptide ZZ were generated and isolated from Hek293T cells using ultrafiltration and bead-elute chromatography. hFc-Ago2 fusion proteins were expressed in Hek293T cells and isolated by affinity chromatography, following which the fusion proteins were incubated in a molecular excess of siRNAs against cyclin D overnight. Excess siRNAs were removed by a second round of affinity purification. $5x10^6$ loaded EVs were co-incubated with 10^5 U2OS cells overnight, following which after 48 hours cells were harvested and cyclin D levels assessed. For the in vivo studies, $1x10^6$ of A549 cells were mixed with 1:1 ratio of Matrigel and injected subcutaneously into NCRNU mice. Tumor-bearing mice were treated every other day with 10^7 siRNA-loaded exosomes using tail-vein administration. Over the course of the study caliper measurements were used to calculate tumor volume. Cyclin D levels following U2OS cell treatment with siRNA-loaded Ago2 displayed on the surface of Lamp2b-ZZ domain EVs showed significant target silencing, as shown in Figure 11. siCyclin D were directly transfected for the +ve control. hFc denotes exosome surface display; siCyclin – anti CyclinD siRNA; siScr – scrambled sequence. n=3.

Claims

1. An EV comprising at least one fusion protein, wherein the at least one fusion protein comprises at least one Fc binding polypeptide fused to at least one exosomal polypeptide.
2. The EV according to claim 1, wherein the at least one Fc binding polypeptide is selected from the group comprising Protein A, Protein G, Protein A/G, Protein L, Protein LG, Z domain, ZZ domain, human FCGRI, human FCGR2A, human FCGR2B, human FCGR2C, human FCGR3A, human FCGR3B, human FCGRB, human FCAMR, human FCERA, human FCAR, mouse FCGRI, mouse FCGRIIB, mouse FCGRIII, mouse FCGRIV, mouse FCGRn, SPH peptide, SPA peptide, SPG2, SpA mimic 1, SpA mimic 2, SpA mimic 3, SpA mimic 4, SpA mimic 5, SpA mimic 6, SpA mimic 7, SpA mimic 8, SpA mimic 9, SpA mimic 10, Fcγ mimic 1, Fcγ mimic 2, and any combination thereof.
3. The EV according to any one of the preceding claims, wherein the at least one Fc binding polypeptide comprises more than one Fc binding region.
4. The EV according to any one of the preceding claims, wherein the at least one exosomal polypeptide is selected from the group comprising CD9, CD53, CD63, CD81, CD54, CD50, FLOT1, FLOT2, CD49d, CD71, CD133, CD138, CD235a, ALIX, Syntenin-1, Syntenin-2, Lamp2b, TSPAN8, TSPAN14, CD37, CD82, CD151, CD231, CD102, NOTCH1, NOTCH2, NOTCH3, NOTCH4, DLL1, DLL4, JAG1, JAG2, CD49d/ITGA4, ITGB5, ITGB6, ITGB7, CD11a, CD11b, CD11c, CD18/ITGB2, CD41, CD49b, CD49c, CD49e, CD51, CD61, CD104, Fc receptors, interleukin receptors, immunoglobulins, CD2, CD3 epsilon, CD3 zeta, CD13, CD18, CD19, CD30, CD34, CD36, CD40, CD40L, CD44, CD45, CD45RA, CD47, CD86, CD110, CD111, CD115, CD117, CD125, CD135, CD184, CD200, CD279, CD273, CD274, CD362, COL6A1, AGRN, EGFR, GAPDH, GLUR2, GLUR3, HLA-DM, HSPG2, L1CAM, LAMB1, LAMC1, LFA-1, LGALS3BP, Mac-1 alpha,

Mac-1 beta, MFGE8, SLIT2, STX3, TCRA, TCRB, TCRD, TCRG, VTI1A, VTI1B, other exosomal polypeptides, and any combinations thereof.

5. The EV according to any one of the preceding claims, wherein the at least one Fc binding polypeptide is displayed on the outer surface of the EV.
6. The EV according to any one of the preceding claims, wherein the at least one Fc binding polypeptide is bound to at least one Fc containing protein.
7. The EV according to any one of the preceding claims, wherein the EV has bound to it a plurality of Fc containing proteins through interaction between the Fc binding polypeptide and the Fc domains of the plurality of Fc containing proteins, wherein the plurality of Fc containing proteins may be the same or different.
8. The EV according to any one of the preceding claims, wherein the at least one Fc containing protein is an antibody and/or a protein engineered to comprise an Fc domain.
9. The EV according to claim 8, wherein the antibody is a targeting antibody, a therapeutic antibody, an antibody-drug conjugate (ADC), or an antibody for reducing opsonization and/or immune cell-mediated clearance.
10. The EV according to any one of claims 6 to 9, wherein the EV having bound to it at least one Fc containing protein is having bound to it at least 10, preferably at least 20, even more preferably at least 30 Fc containing proteins.
11. The EV as claimed in any one of claims 6-10, wherein the at least one Fc containing protein is present inside the EV, in the lipid membrane of the EV and/or attached to the outer surface of the EV.
12. A non-covalent complex between (i) a fusion protein, and (ii) an Fc containing protein, characterized in that the fusion protein comprises at least one Fc binding polypeptide fused to at least one exosomal polypeptide, wherein the Fc binding polypeptide binds to the Fc containing protein.
13. The non-covalent complex according to claim 12, wherein the non-covalent complex is present in the lipid membrane of an EV and/or inside an EV.

14. A pharmaceutical composition comprising EVs according to any one of claims 1 to 11, and/or the non-covalent complexes according to any one of claims 12 to 13, and optionally a pharmaceutically acceptable carrier.
15. The EVs according to any one of claims 1 to 11, and/or the non-covalent complexes according to any one of claims 12 to 13, and/or the pharmaceutical composition according to claim 14, for use in medicine.
16. A method for attaching to an extracellular vesicle (EV) at least one Fc containing protein, comprising the steps of:
    (i) providing an EV comprising a fusion protein comprising at least one Fc binding polypeptide and at least one exosomal polypeptide;
    (ii) allowing the Fc binding polypeptide of the fusion protein to bind the Fc domain of at least one Fc containing protein.
17. A polypeptide construct comprising a fusion protein comprising at least one Fc binding polypeptide and at least one exosomal polypeptide.
18. A polynucleotide construct encoding for at least one polypeptide construct of claim 17.
19. A cell comprising at least one polypeptide construct according to claim 17, at least one polynucleotide construct according to claim 18, at least one EV according to any one of claims 1-11 and/or at least one non-covalent complex according to any one of claims 12-13.
20. A method for delivery of at least one Fc containing protein to a target cell, comprising (i) attaching the Fc domain of the at least one Fc containing protein to an Fc binding polypeptide comprised in an EV, and (ii) contacting the EV, having attached to it the at least one Fc containing protein, with the target cell.
21. The method according to claim 20, wherein the method is carried out in vitro, in vivo, and/or ex vivo.
22. The method according to any one of claims 20-21, wherein the Fc binding polypeptide of the EV is part of a fusion protein with at least one exosomal polypeptide.

23. The method according to any one of claims 20-22, wherein the Fc containing protein is delivered into a target cell.